



Center for Toxicology, Southwest Environmental Health Sciences Center (an NIEHS Center)

Experimental Pathology Core

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<http://swehsc.pharmacy.arizona.edu/exppath/>

SWEHSC Science fair is May 15, 2000:

The annual science fair is being held at the Westward Look Resort (245 E. Ina Rd). The scientific sessions begin in the conference center at 1 pm. The poster session will be on the Watson Terrace and it begins at 3:30 pm. Contact Bert Sanchez (626-2433) for more details.

Web site move:

The SWEHSC is moving its entire web site to a new server. The site has been completely re-designed so that the pages have a cohesive look and site navigation is greatly improved. While things are still early in the process of being moved over to the new site, visit <http://swehsc.pharmacy.arizona.edu/> and see how things are progressing. The Experimental Pathology Core portion of the site will feature an expanded on-line brochure detailing the many ways we can assist SWEHSC investigators with their research.

Appropriate use of quantitation with fluorescent microscopic images:

In the past number of months there has been some confusion within the SWEHSC regarding the appropriate use of quantification with microscopic images. In particular there has been an interest in quantitating the fluorescence images generated by the confocal microscope. We have studied this topic, read books & articles, asked many questions and tried to come to a common understanding among ourselves about how to approach this topic. What follows is what we feel to be an appropriate and scientifically valid approach to this topic.

To begin, it would be appropriate to clarify what we mean by quantitation:

- We are not referring to standard morphometric techniques for the measurement of tissue structures. There is nothing controversial about performing area, perimeter, and length type measurements on confocal or other widefield fluorescence microscopic images.
- Within this context we are defining quantitation as the measurement of *relative* intensity values of large areas that have been prepared and acquired under fairly specific conditions.
- We do not wish to imply that quantitation is the same as densitometry (*measurement of small changes in intensity values with statistically appropriate precision by using calibrated reference standards and/or correlating these values with specific quantities of proteins, DNA or enzyme activity within cells or groups of cells*). Densitometry is difficult to perform in almost any experimental system. With the confocal there are a large number of variables that are extremely difficult to control that make it almost impossible to acquire this level of statistically valid data.

It would require a lengthy review article to fully explain all of the factors involved in making densitometry of confocal images an inappropriate choice. James Pawley, PhD, editor of the [Handbook of Biological Confocal Microscopy](#) and director of the course "3-D Microscopy of Living Cells" has developed a list of 39 items that must be adequately controlled for, or understood, before it would be appropriate to perform densitometry on confocal images.

As an example, two of the items on the list that specifically relate to the instrument include:

- poisson or "shot" noise
 - Random variations in the illumination that can be fairly significant at low light levels

- laser instabilities
 - our confocal has no accurate means for measuring the power output at a given wavelength
 - Lasers are typically unstable for the first 1-2 hours after they are turned on
 - Air cooled lasers can be sensitive to small changes in room temperature

A few of the items on the list that relate to the specimen include:

- optical issues related to the coverslip and mounting media
 - Our confocal microscope's optics require a #1.5 thickness coverslip
 - Use of a coverslip of incorrect thickness and/or mounting media that has a refractive index that is not close to the RI of glass [1.5185] will increase the degree of spherical aberration in the image, with fluorescence this artifact can cause light to appear in places that are removed from its actual source
- local dye concentration
 - If the dye has a specific concentration within a cellular compartment and the compartment changes size then the intensity will appear to have changed
 - An example of the effects of changes in cell volume and/or hydration states, with a concomitant increase in "apparent" concentration of proteins, is a cell undergoing apoptosis. Comparison of the measurements of relative intensity values of protein stains in normal and apoptotic cells may not reflect true alterations in protein expression or stability. This is because apoptotic cells are known to undergo dehydration and shrinkage.
 - In instances where the dye is highly concentrated in a small cellular compartment, the formation of excimers can cause some of the dye to fluoresce at a longer wavelength thus causing the intensity at the original wavelength to decrease
- photobleaching
 - Confocal users quickly learn that the intense laser light fades their specimens
 - In order to make any kind of comparison the exposure of the specimen to the excitation wavelength must be uniform for each image field

We recognize that this causes a problem for investigators who are interested in demonstrating the significance of the changes that their experimental protocols have caused. This is compounded by the fact that journals do not always reproduce images with sufficient accuracy to demonstrate subtle changes in intensity. **As with most microscopy-related problems, we urge SWEHSC investigators to consult with the Experimental Pathology Core about appropriate ways to acquire and measure microscopic data.**

An example of careful quantitation:

Dr. Claire Payne's research has incorporated the measurement of confocal images to determine relative intensity values. Dr. Payne uses these values to demonstrate differences between experimentally treated and control cells. While she does occasionally use statistical analyses on the data, often she simply reports the differing levels of intensity as a means to verify the observed differences in the images. This is most useful when comparing a large number of images. Journals frequently recommend that the author provide images that are representative of the study's findings. A table of relative differences in intensity values allows the author to tabulate all of their observations. **Under no circumstances do relative intensity values reflect actual amounts of the proteins or other molecules of interest.**

What may not be obvious about Dr. Payne's use of this technique is her VERY meticulous attention to detail in preparing the specimens and acquiring the images. This attention to the fine details is crucial to ensuring the validity of the data.

Here is a listing of the kinds of things Dr. Payne is controlling for:

- Fixation and processing of the tissue rigorously follows a specific protocol, and the treated & control groups are fixed & processed at the same time, using the same reagents.

- The protocols for antibody markers are followed explicitly and the reactions for pairs of control & treated specimens that will be analyzed are performed at the same time using the same batch of reagents.
- Dr. Payne has acquired images 15 minutes after the laser has been turned on and again two hours later. Her studies indicate that any instability in the laser power does not seem to produce statistically significant variations in the measured image intensity values.
- Dr. Payne uses two fluorescent dyes. The first is a nuclear stain called YOYO-1 (*on RNAase-digested samples this then becomes a DNA-specific stain*) that has similar excitation/emission characteristics to Fluorescein (YOYO-1 – Ex_{max} 491 nm, Em_{max} 509 nm). The second dye is CY-5 that is conjugated to a secondary antibody (CY-5 – Ex_{max} 649 nm, Em_{max} 666 nm).
- Dr. Payne uses the Leica confocal's 488 nm laser line to image the cell nuclei and focus the microscope. This technique has several advantages. It satisfies the need for random sampling since the fields are not pre-selected based on the CY-5 staining (*the stain that she measures*). An additional advantage is that when she acquires her images of the CY-5 staining, the dye has not been exposed to the appropriate excitation wavelength; therefore it has not had a chance to photobleach. Using the YOYO-1 staining, she can adjust the microscope's fine focus controls such that the image represents the brightest plane of focus in the section. When she does acquire her CY-5 images she turns the laser to its maximum power setting (*the only semi-reproducible power setting on the laser*). The photomultiplier settings for the CY-5 image are determined using an area of the section that is not included in the actual data acquisition and are saved for reuse throughout the session. She makes sure that her image does not oversaturate the confocal's photomultiplier since oversaturated images cannot be meaningfully quantitated.
- Once she has made the appropriate adjustments to the instrument settings (*specific filter sets, specific magnification, specific settings for image averaging, laser power at maximum, specific photomultiplier and pinhole settings*), these are not changed during the acquisition time frame. Both the control and treated samples are examined within a short time frame (*typically 15 min*) to avoid any significant drift in the laser power.
- Dr. Payne has tested this procedure by alternating her acquisitions back and forth between treated and controls. Her intensity values are consistent between different areas of the slide, with an acceptable standard deviation.
- Dr. Payne's actual intensity measurements (*sometimes expressed as "gray values" since the 8-bit detector can measure 256 different intensities or gray values*) are taken from a field of cells that has been outlined in her image analysis program. The mean intensity value of the field (\pm S.D.) is averaged with similar measurements in treated and control tissues and the final means are compared to demonstrate the differences in intensity.