Zoological Studies 34, Supplement I (1995)

Zoological Studies

On-line Attenuation Compensation in Confocal Microscopy

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The ability to perform optical section with a confocal laser scanning microscope, provides great opportunity to take images at different depths within a sample. But images obtained from deep into the sample will also have longer optical paths, which will have lower signal intensities because of absorption and scattering by the sample 1. This gradual signal attenuation at increasing optical path will reduce S/N ratio and dynamic range of the image stack. The registration of similar structure with reducing intensity will also create difficulty in subsequent analysis such as image segmentation and three dimensional reconstruction. A heuristic method is developed to compensate this attenuation effect.

There is several parameters can be adjusted during the imaging process to get optimal images. The supplied voltage to photomultiplier tube (PMT) is a primary one to control the amplification of incoming signals. Since the gain of a PMT is a function of the supplied voltage, when taking an image stack from a thick sample, it is possible to change the supply voltage of the PMT to modify its amplification, and thus compensate signal attenuation at different depth. The output signal of a confocal microscope can be shown as:

$$I = L \times G\ (\ Vpmt)$$

where L is incoming light detected by the PMT, G is the total amplification of the electronic system, Vpmt is the supplied voltage to PMT and I is the output.

If we add appropriate compensation voltage (Vcom) to the PMT supplied voltage (Vpmt), it will increase or decrease the amplification on detected signals. This will bring output signals from images at difference depth to a similar intensity level. Therefore, the intensity of an image at step i in an image stack can be expressed as:

$$I(i) = L(i) \times G(\ Vpmt + V(i)com\ )$$

Then, the task is to select proper intensity representation of the images, and calculate the compensation voltage base on them. One method to achieve this is to let the microscope user to select similar features (e.g. cell nuclei) in images obtained at different depth, and derive the compensation voltage from the images. Gererally, the features of interest in an fluorescent image are composed of pixels with high signal levels. Therefore, in an automatic approach, the mean of a certain range of bright pixel value can be used to calculate the compensation voltage.

Our heuristic algorithm to compensate attenuation in an imaging stack can be summarized as:

1. The relationship between supplied voltage of the PMT and signal intensity is measured from a homogeneous sample of certain fluorescent dyes such as 0.1% FITC in DMSO solution.
2. At the first slice (the slice closes to the objective) of an image stack, proper illumination intensity and PMT voltage are selected in order to obtain optimal image and with enough adjustment range of the PMT voltage for later compensation. Acquire the image and calculate the mean value of the brightest group of pixels.
3. Move the specimen to the position of last slice (the deepest slice) and repeat step 2 with a proper voltage setting.
4. Use the differences between the mean values from step 2, 3 and data from step 1 to interpolate for compensation voltage.
5. Start the sequence of acquiring a stack of serial optical sections by adjusting the PMT voltage as a function of the sectioning depth based on the interpolated result.

A maize stem, fixed in a1:3 acetic acid/EtOH fixative, dehydrated in EtOH and cleared in winter

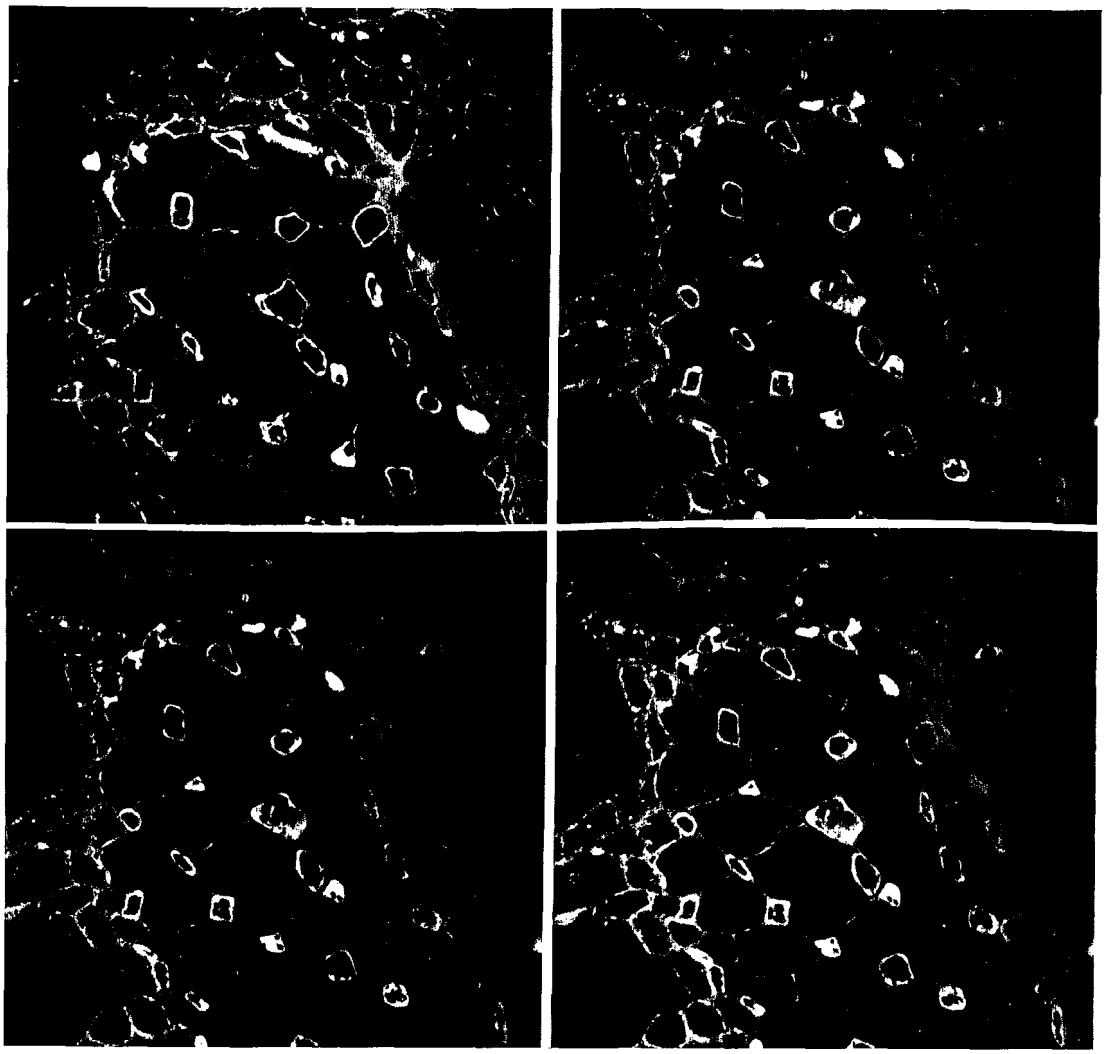

Fig. 1. Cross section of a maize stem. 1A: near the top surface of the sample; 1B: optical section obtained at a depth of 42um from the section shown in 1A; 1C: optical section obtained at the same position as 1B but with proper compensation; 1D: optical section obtained at the same position as 1B but with over compensation.

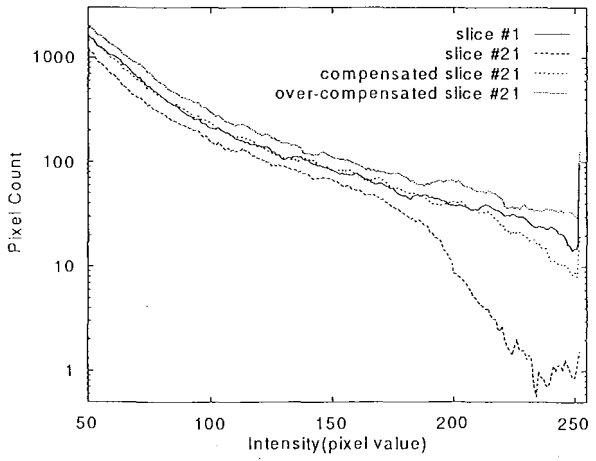

Fig. 2. Intensity histogram of image 1B, 1C and 1D.

green oil, was used as the test sample. The 488nm Ar ion line was used as the excitation wavelength and a 515 nm longpass filter was used in the detecting path. The image in Fig. 1A was taken near the top surface of the sample (first slice). Figure 1B shows the 21st slice which was 42μm deeper than the first slice. Figs. 1C and 1D were also taken at the same position as Figure 1B, but with proper compensation and overcompensation (2X of compensation voltage). The intensity histogram of these four images are plotted in Fig. 2. It clearly demonstrates the compensation method was able to move the histogram of an attenuated image obtained from deeper slice up to a similar level in comparison to the first slice.

This intensity compansation process is incorporated into the control software (developed in our lab at SUNY) for Olympus GR-200 laser scanning confocal microscope running under Microsoft Windows.

REFERENCE

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