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Letter to Editor

Quantitative immunohistochemistry by measuring chromogen signal strength using a C# written program

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Immunohistochemistery (IHC) is increasingly being used for diagnostic purposes. It is also a frequently used technique in both clinical and basic research to identify and map the distribution of specific antigens. [1] Traditionally these techniques rely solely on the subjective visual estimation by an expert and yield qualitative results. [2] The comparison of staining among sets of tissues requires some form of quantification. Indeed, analysis using a quantitative methodology of immunostained specimens provides more information and leads to a more accurate elucidation of putative physiological and pathophysiological results.[1] Different labs and research centers have used their own home developed computer software of image processing appliances to get more objective and observer independent methods.[3]

We developed IQ.IHC software that quantify the staining strength in digital images of diaminobenzidine (DAB) stained IHC samples using C# language (Microsoft Visual Studio 2005 and Dot Net framework 2.0).

After taking digital images of samples in controlled optical conditions, up to 10 segments of the sample image should be manually selected. Pixels containing the chromogen were detected using a thresholding method incorporating the red, green and blue (RGB) values. The threshold was defined by asking several experts to mark the stained area and then changing the threshold to maximize the overlap of the expert selected area with the pixels above the threshold. Only for pixels passing the threshold, we measured the strength of the chromogen. In order to overcome the noises and inter- and intra-assay variations, we selected a background segment from white space of slides for each sample. The chromogen strength of background was subtracted from each specimen. The color intensity of each sample was calculated as average of red, green and blue channels of all 10 segments.

We provided visual feedback for the experimenter to check both the background removal efficacy and the distance from the maximum stained point. This helped the experimenter to manually adjust the parameters in the software to gain the best results (Figure 1).

Also to check if the software was producing reliable results and to see if the manual selection of samples by the experimenter could adversely affect the measurement reliability, we performed a "boot strap" [4] procedure with thousands of automatic random re-sampling of a set of IHC digital images. In order to rebut the possibility of observer bias (effect of manual selection of the regions of interest) and test the reliability of the algorithm, we used an automatic randomized region of interest selection method. As depicted in figure 2, we repeated random sampling for 2000 times and selected the maximum calculated signal strength as the estimated chromogen signal strength. We showed that there was a strong correlation between estimated chromogen signal strength and manually measured signal strength (Pearson's correlation coefficient = 0.88, $p < 0.001$).

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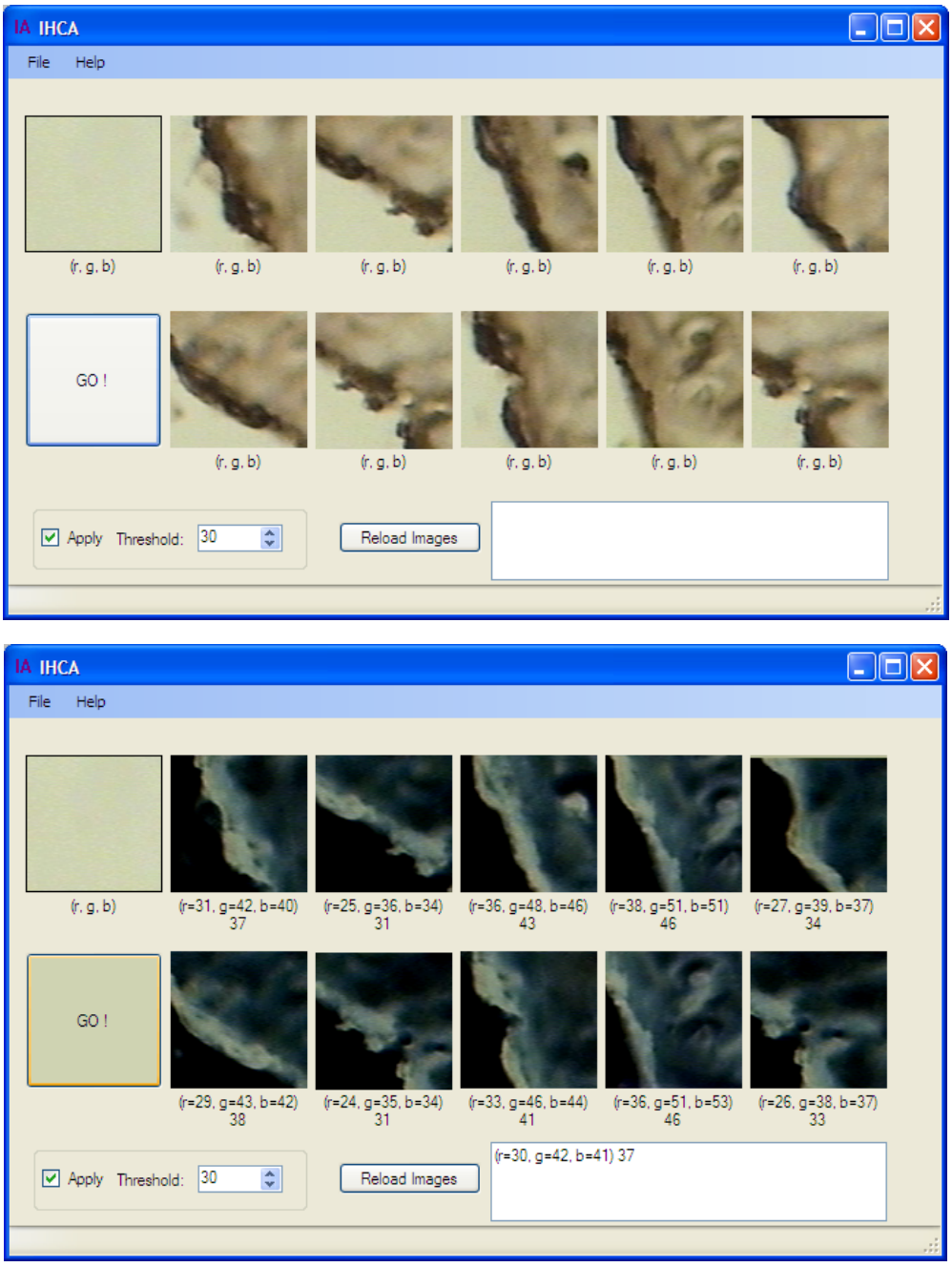

Figure 1. A preview of IQ.IHC software

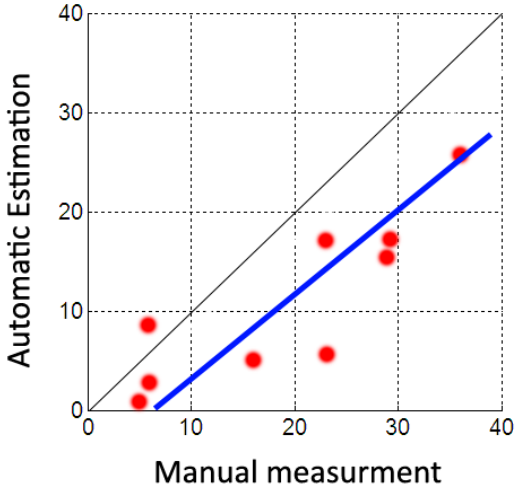

Figure 2. To check the reliability of software we performed a "boot strap" procedure with thousands of automatic random re-sampling of nine IHC digital images. There was a highly significant correlation between the chromogen concentration values estimated by the experimenter using the software (manual estimation) and the values estimated through the resampling method (Automatic estimation). Abscissa and ordinate are both showing the number of chromogen containing pixels (adjusted to the count of pixels with maximum chromogen concentration). Each red dot in the graph shows one of the IHC samples. The thin line shows the ideal case and the thick line shows a linear fit to the real cases.

Conflict of Interest

Authors have no conflict of interests.

Authors' Contribution

ShHJ designed the project and did tissue processing and IHC staining. AM wrote the software, optimized it and checked the reliability. Both authors analyzed the results and wrote the paper.
All authors have read and approved the content of final manuscript.

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