Anatomy, Histology and Color Thresholding

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For over 5 years, here in Pathology at Tulane, I have used color thresholding (CT) rather than monochrome thresholding (MT) for objective quantitation of histological, immunohistological[5], fluorescent and thick TEM sections[7]. Technical aspects distinguishing CT from MT were recently described in detail[2], and this technical note represents an abbreviation of the last paper, which I recommend for those beginning to use video imaging technology. The system I use is the V150 (r) from ONCOR. Despite the advantages of today computers, data outcome, e.g., unbiased, still depends on good experimental design, strict control and repeatability. When the stain of interest is histochemically derived, my laboratory follows standard procedures found in the *Handbook of Histopathological and Histochemical Techniques*[1]. When the stain is immunohistochemically derived, my laboratory checks cross reactivity of every antibody with known positive and negative control tissues besides using irrelevant antibodies or omitting the primary antibody. In addition, preabsorbing the antibody is done whenever its substrate is available[4].

Repeatitive quantification: The main objective of using image analysis should be to repeat measures, reduce errors and avoid bias. It is rather easy to generate large amounts of meaningless data. Two types of quantifications can be done with CT: 1) Relative concentration of immunoreaction, by determining the saturation (% transmission) of pixels representing the stain of interest, and 2) color ratio of positive vs. negative structures, by determining the surface area of immunostained cells (positive pixels) over the counterstain or background color. CT takes into account both the saturation of the color and the density it represents. Most importantly, filtration is not needed to separate colors. This is very important because in a black and white image, a dark blue hue (e.g., hematoxylin) can have the same density as the brown or red hue of an immunoreaction[5]. Slides are viewed directly on a microscope. The reaction product color and intensity are set by numerical thresholds, pixels within such values are turned on and the % transmission is determined[2]. Numerical values are exported to a spread sheet and statistical analysis done[4].

In situ hybridization and dark field: With specific nucleic acid probes, in situ hybridization is straightforward[8]. It is similar to immunohistochemical procedures[9], but usually requires modifications of dilution, temperature, incubation time, stringency and blocking procedures, etc.[3,4] Separation of adjacent pixels of related hues from these preparations is difficult with MT, in which 256 possible levels of separations are reduced to 128 possible comparative steps. CT is based on millions of differences, permiting wider separation spread between pixels.

Data Analysis: Color thresholding allows fine tuning to hue variations of reaction products' color (e.g., brown diaminobenzidine, red phosphatase, blue hematoxylin counterstain, etc.). The mean values of measurement are plotted and the results presented in graphic form with standard error mean or standard deviations[2,5].

The system separates primary and secondary colors based on Hues-Saturation-Intensity (HSI), allowing a theoretical 16 million color possibilities (Figures 1-2). Differences between adjacent pixels are not always easy to separate with monochrome systems because they often cannot separate hues (Figures 3 - 4) of similar intensities (e.g., a dark brown and dark blue). This is particularly important when trying to count particles (gold, isotope) in a dark background (e.g., dark field and in situ hybridization). The HSI mode is based on the same principle used by the human brain to distinguishes colors. It consists of a three dimensional space wherein discrimination of colors is performed without the need of filter selection. This is important because filters only select for hue and not for saturation. Pixels that are impossible to separate with a monochrome system (as those encountered in dark field imaging), are effortlessly separated with color thresholding in real time. ■

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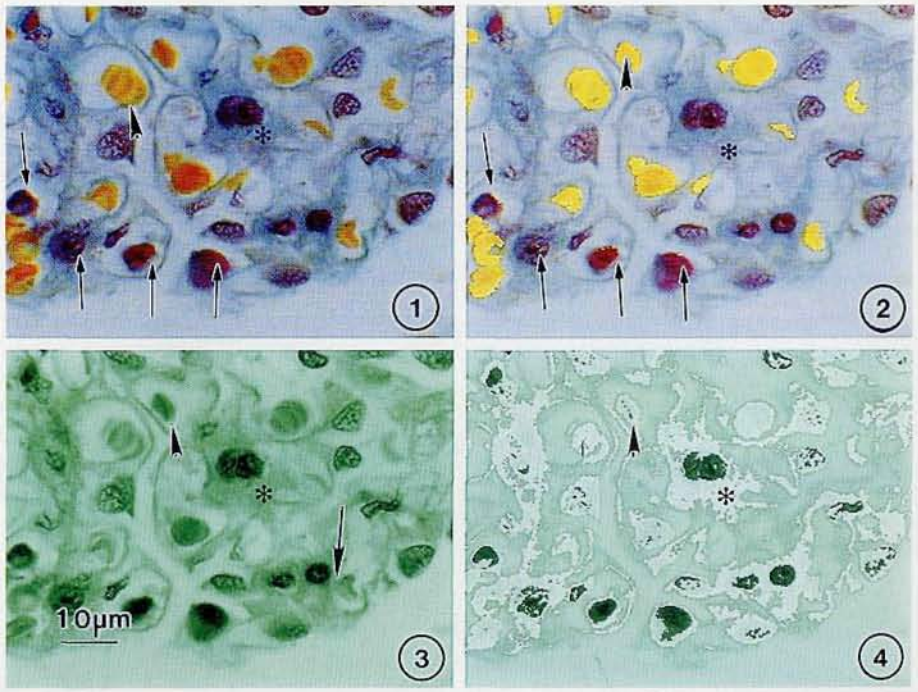

Figure 1. True color image of a glomerulus polychrome-stained (Shoobridge, 1983) with total separation of hues representing each color. A mesangial cell (*) is clearly separated from other structures based on hues not their densities. The sensitivity of CT is shown here by clear separation of yellow color around re-stained lymphocytes (arrows), and the yellow-stained red blood cells (arrowhead) are not grouped with other structures of similar density.

Figure 2. Pixels representing red blood cells (arrowhead) are turned on selectively, including those near the red-stained lymphcytes (arrows). Note however, that the mesangial cell (*) is not chosen.

Figure 3. A monochrome representation of the same area at 24 bit resolution showing that different colors usually have similar densities and are grouped (thresholded) so that yellow-stained red blood cells (arrowhead) and parts of the mesangial and epithelial cells (arrow) are difficult to discern.

Figure 4. When the same CT values used in figure 2 are use in a monochrome mode, pixels representing the hues (now densities) of the mesangial (*) and epithelial cells are grouped with the red blood cells (arrowhead). Excluding pixels from un-intended structures makes quantification more accurate, and objective, with the HSI than with monochrome thresholding.