

TECHNICAL NOTE

Quantitative Immunohistochemistry by Measuring Cumulative Signal Strength Using Commercially Available Software Photoshop and Matlab

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SUMMARY Currently available techniques for performing quantitative immunohistochemistry (Q-IHC) rely upon pixel-counting algorithms and therefore cannot provide information as to the absolute amount of chromogen present. We describe a novel algorithm for true Q-IHC based on calculating the cumulative signal strength, or energy, of the digital file representing any portion of an image. This algorithm involves subtracting the energy of the digital file encoding the control image (i.e., not exposed to antibody) from that of the experimental image (i.e., antibody-treated). In this manner, the absolute amount of antibody-specific chromogen per pixel can be determined for any cellular region or structure. (J Histochem Cytochem 48:303–311, 2000)

KEY WORDS

bombesin
cancer
gastrin-releasing peptide
methods

Quantitative immunohistochemistry (Q-IHC) in the predigital era depended on the observations of multiple investigators using an arbitrary scale to grade the extent and intensity of chromogen present (Shi et al. 1991,1993). This approach was inherently limited by observer subjectivity and bias, by inter- and intra-observer variation, and generated data of limited range (i.e., amount is usually quantified on a 0–4 scale). With the advent of digital photomicroscopy, however, these weaknesses could in theory be eliminated and true quantification achieved.

Early attempts at Q-IHC involved converting analog images into a digital format and then transforming the 256 separate shades of red, green and blue that are obtained when working in 24-bit RGB color to single-channel grayscale (Mosedale et al. 1996). The area of interest is defined and the mean gray level of the selected pixels determined. Later attempts at Q-IHC employed color thresholding using commercial software (i.e., Adobe Photoshop) (Fermin and Degraw 1995), followed by counting the total number of pixels of

appropriate value (Lehr et al. 1997,1999; Ruifrok 1997). For example, in DAB-based immunohistochemistry all the pixels containing “brown” within a pre-specified spectral range are identified in Photoshop using the Magic Wand tool. The total numbers of pixels identified are then counted using the histogram function (Lehr et al. 1999). Although these algorithms represent significant improvements over traditional methods for evaluating analog images, these approaches still do not allow true Q-IHC to be performed.

Determining the absolute amount of chromogen present necessitates calculating the cumulative signal strength of the image being evaluated. This can be done only by calculating signal energy, E , which is defined in the mathematical (Jain 1989) and not the physical sense. Here we provide an algorithm for true Q-IHC that relies on calculating the energy of images captured in Photoshop using a high-resolution digital camera and then processing the image’s unmodified and full digital file using the powerful enabler language Matlab. To demonstrate this algorithm, we studied the gastrin-releasing peptide receptor (GRPR) aberrantly expressed by human colon cancers. Because we have previously shown that human colon cancers variably express this receptor as a function of tumor differentiation (Carroll et al. 1999), this tissue type rep-

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resents a good model for demonstrating the enhanced power of digital quantitative immunohistochemistry.

Materials and Methods

Reagents

Antibody against the GRPR (Kroog et al. 1995) was obtained from Dr. James Battey (NIDCD, NIH; Bethesda, MD). All immunohistochemical reagents, including Large Volume DAKO LSAB(R)2 Kit and DAKO Liquid DAB Substrate-Chromogen System were from DAKO (Carpinteria, CA). All other reagents were obtained from Sigma (St Louis MO). All reagents and solvents were used at reagent grade purity.

Immunohistochemical Technique

Four separate colon cancers were selected from the the Chicago Veterans Administration Medical Center Gastrointestinal Tissue Bank representing a broad spectrum in terms of tissue differentiation. Differentiation was assessed by standard criteria as previously defined (Anonymous 1992). Immunohistochemistry was then performed using a standard three-stage indirect immunoperoxidase technique on 5- μ m tissue sections (Jasani and Schmid 1993). Briefly, fixed tissue sections were rehydrated in graded alcohols and then rinsed in a running water bath, with endogenous peroxidase activity quenched by preincubating slides in 3% hydrogen peroxide in a light-impermeable chamber. After washing in PBS, slides were incubated for 20 min in blocking solution [5% skim milk (v/v) and 0.15% H₂O₂ (v/v) in deionized water]. The slides were washed in PBS, primary antibody applied at a 1:250 dilution, and the tissue incubated for 1 hr in a humidity chamber. After washing again in PBS, the tissues were incubated with biotinylated anti-rabbit IgG (DAKO) for 15 min. After washing in PBS, the slides were incubated with streptavidin conjugated to horseradish peroxidase (DAKO) for 15 min and washed again in PBS buffer. Incubating slides with Liquid DAB Substrate-Chromogen System (DAKO) for the indicated period of time (2 min unless otherwise specified) identified bound antibody. After a final wash in PBS and distilled water, the slides were counterstained with a 50% dilution of Gill's hematoxylin for the indicated period of time (usually 1 min unless otherwise specified), dehydrated in graded alcohols, and mounted with a coverslip using Permount.

For all specimens, control tissues were processed identically and at the same time, except that primary antibody was not applied. Control tissues were within 5 μ m of the tested tissue and treated with secondary antibody, DAB, and counterstained with hematoxylin for precisely the same time as the experimental tissue. Therefore, all differences between the experimental tissue and the control tissue are ultimately due to DAB identification of the relevant protein.

Digital Image Capture

All specimens were evaluated using a Nikon E600 microscope with Axioplan objectives. Images were acquired at $\times 1000$ using a Microlumina Ultra Resolution Scanning Digital Camera [3380 \times 2700 pixels (Leaf Systems; Fort Washington, PA)]. In all cases, files were saved in tagged-image

file format (TIFF), which allows LZW compression without discarding any data, as occurs when the more popular joint photographic experts group (JPEG) format is used. Consequently, the average file size of each original digital image was 22–25 MB.

Determination of Image (File) Energy

We assume that all information contained in the experimental image file (i.e., from the primary antibody-exposed slide), minus the information contained in the control image file (i.e., from the slide not exposed to primary antibody but otherwise treated identically), is of importance. In other words, the amount of antibody-generated chromogen is represented by all the information contained in the experimental image file, minus that contained in the control image file. In this manner we make use of all the numerical data encoded within the file describing each pixel.

After acquisition with a digital camera, the file for the experimental image is opened in Photoshop (Adobe; San Jose, CA) using a Macintosh 400 MHz G3 workstation (Apple; Cupertino, CA). The region of interest is defined in Photoshop using the Marquee tool. This tool allows an area of any pixel dimension to be determined by the operator by using the "options window." To do this, the appropriately sized region of interest is identified and stored in a new file. This subset of the experimental image is labeled "EXP" and saved in TIFF format, as follows.

Overview of the algorithm for determining cumulative signal (i.e., image) intensity

1. In Photoshop
 - A. Open Image
 - B. Define size parameters for Marquee tool
 1. Double click Marquee icon
 2. Define area in pixels
 - C. Identify region to be analyzed with Marquee tool
 - D. Store in new file
 1. Function "Edit" select "Copy"
 2. Function "Edit" select "New File"
 3. In the new file select "Paste"
 4. Function "Save" select tiff for file type
 - E. Store new file in Matlab folder

2. In Matlab

```
At the cursor (>>) type
>>a=double(imread('EXP','tiff'));
>>b=double(imread('CONTROL','tiff'));
>>ea=norm(a(:,1))+norm(a(:,2))+norm(a(:,3));
>>eb=norm(b(:,1))+norm(b(:,2))+norm(b(:,3));
>>et=abs(ea-eb);
>>en=sqrt(et^2/N)
```

Note:

1. "double" allows double floating point precision for all calculations
2. "imread" loads an image into the program
3. "norm" calculates the singular value of a matrix
4. "abs" takes the absolute value
5. "sqrt" calculates the square root
6. Semicolons after each line of code prevent the program from running after entering a new line of instruction

7. "N" in the last line of code, refers to the number of pixels contained within the image being evaluated, or 10,000 for a 100×100 pixel image.

The file for the control image is processed similarly. The control slide is acquired from a sequential 5- μm tissue section and is treated identically to the experimental slide, except for not being exposed to antibody. The same area that was studied in the experimental situation is identified. The Marquee icon is used at the same size setting as was used for the experimental image and is placed over the homologous region in the control image. As above, the selected region is cut out and stored in a new file in TIFF format. This subset of the control image will now be referred to as "CONTROL." For ease of subsequent operation, both files "EXP" and "CONTROL" are stored in the Matlab folder.

Both files "EXP" and "CONTROL" are opened in Matlab (Math Works; Worcester, MA) using the read image command "imread." The data contained within each RGB image file is stored as an $m \times n \times 3$ matrix. Thus, the "norm" function can be used to determine the singular value of the matrix, which represents the magnitude, or mathematical energy, of the image file (the rationale for this algorithm is provided in the Discussion). Finally, the amount of energy present per pixel is determined by taking the square root of the difference of each file's energy squared divided by the number of pixels evaluated (see above). The purpose of computing the square root of the energy of the image file squared, divided by the number of pixels, is to determine the normalized energy of the image file per pixel. This transformation is necessary because the computation of the energy is determined by the square root of the summation of the squares of the image intensity in each pixel. The normalized energy per pixel therefore requires the determination of the square root of the average of the squares of the image intensity in each pixel.

Statistical Analysis

All values reported herein are unit-less. Values are therefore reported as energy units per pixel (eu/pix). Statistical analysis was performed using StatView (Abacus Concepts; Berkeley, CA), with differences between tissue regions evaluated by ANOVA. In all instances, data are expressed as the means \pm SEM.

Results

We have previously shown that whereas epithelial cells lining the human colon do not normally express GRPR (Ferris et al. 1997), this receptor is aberrantly expressed by many but not all human colon cancers (Carroll et al. 1999). Specifically, we have found that increased amounts of GRPR immunostaining are found in well-differentiated colon cancers. Less is seen in moderately differentiated cancers, and none is apparent in poorly differentiated cancers (Carroll et al. 1999). This can be appreciated in Figure 1. When four colon cancer samples with various degrees of differentiation

were processed simultaneously, the grossly apparent amount of GRPR immunostaining varied directly as a function of tumor differentiation (Figure 1).

We first evaluated how large a window was necessary for accurate determination of chromogen per pixel. Specifically, we compared the information obtained using different-sized windows (10×10 pixels, 100×100 pixels, and 200×200 pixels; actual sizes are shown in Figure 2). In all instances, the energy contained within three separate regions was evaluated for each of the three window sizes, thereby enabling us to determine the coefficient of variation between observations. When a tumor containing a large homogeneous amount of cytoplasmic chromogen was evaluated (i.e., Figure 1A), the coefficient of variation between separate regions evaluated at even the relatively small window size of 10×10 pixels was low (Table 1). In contrast, tumors containing less cytoplasm were more dependent on window size. For example, moderately poorly differentiated colon cancer (Figure 1C) on average expressed 55 eu/pix for both the 10×10 pixel region and the 100×100 pixel region. However, the coefficient of variation was \sim sixfold higher for regions evaluated using the smaller window (Table 1). Although the coefficient of variation becomes smaller when windows between 10,000 and 40,000 pixels in size are used to evaluate homogeneous regions, larger window sizes are not better for evaluating non-homogeneous regions (Table 1). For example, the small amount of area devoid of nuclei in poorly differentiated cancers (i.e., Figures 1C and 1D) makes it impossible to place a 200×200 pixel window that fits entirely over cytoplasm (Figure 2, bottom panel). Indeed, the inclusion of noncytoplasmic regions in such a large region is what makes the coefficient of variation larger than that observed when the energy contained in a 100×100 pixel region is determined. The importance of performing Q-IHC on areas that are homogeneous within the window being evaluated is underscored by the results obtained using the 200×200 pixel window on poorly differentiated cancer. Whereas the smaller window sizes indicate that there is ~ 5 eu/pix GRPR in the cytoplasm of poorly differentiated cancers, the necessary inclusion of nuclei dramatically increases this value to >30 eu/pix in the 200×200 pixel window (Table 1). Therefore, these data demonstrate that only homogeneous regions within an image can be evaluated. However, these data also show that windows as small as 10×10 pixels still can provide accurate information, the precision of which is increased by sampling multiple different regions within a specimen. However, all subsequent experiments were performed using 100×100 pixel windows, given the larger size and associated low coefficient of variation independent of the degree of tissue differentiation.

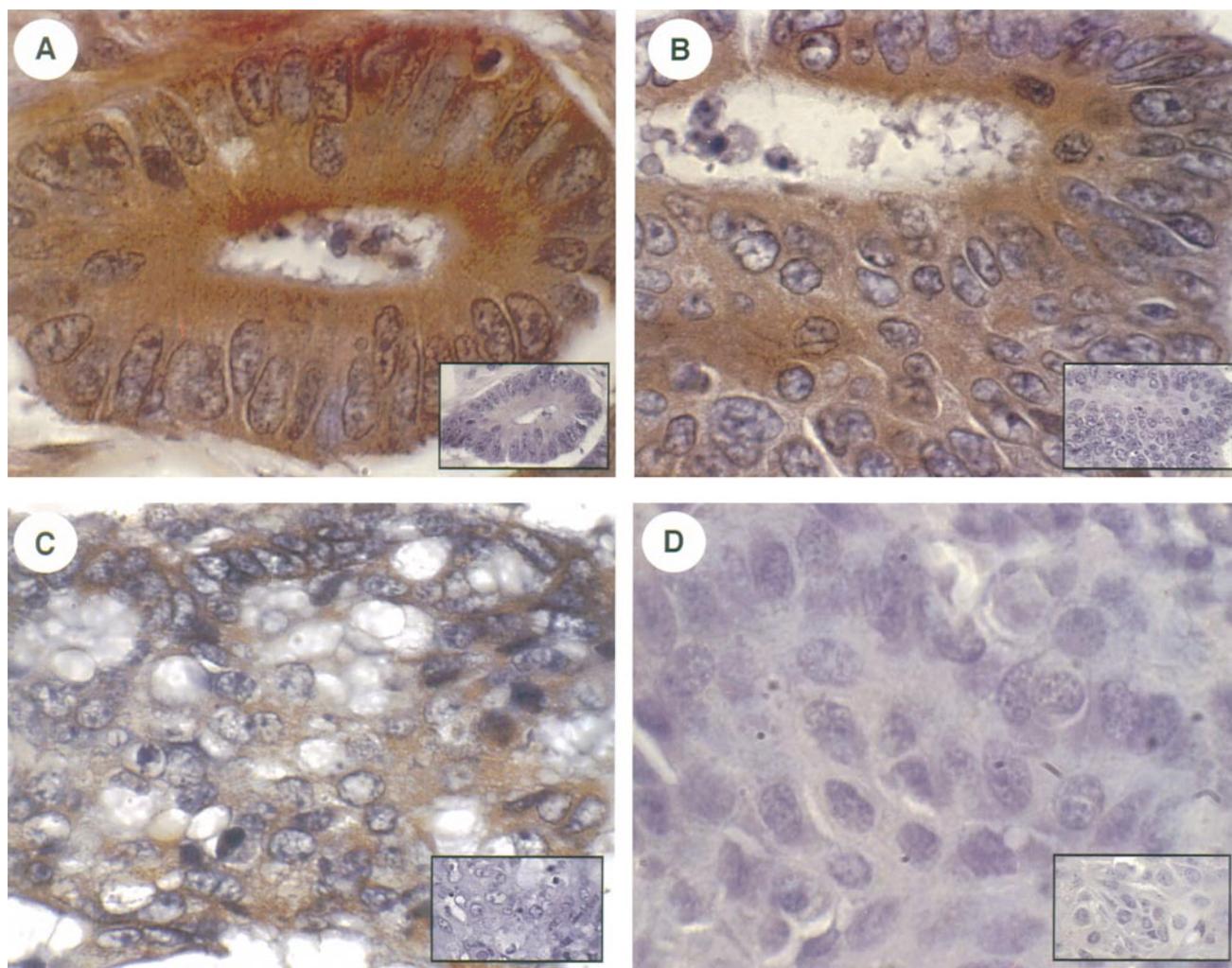


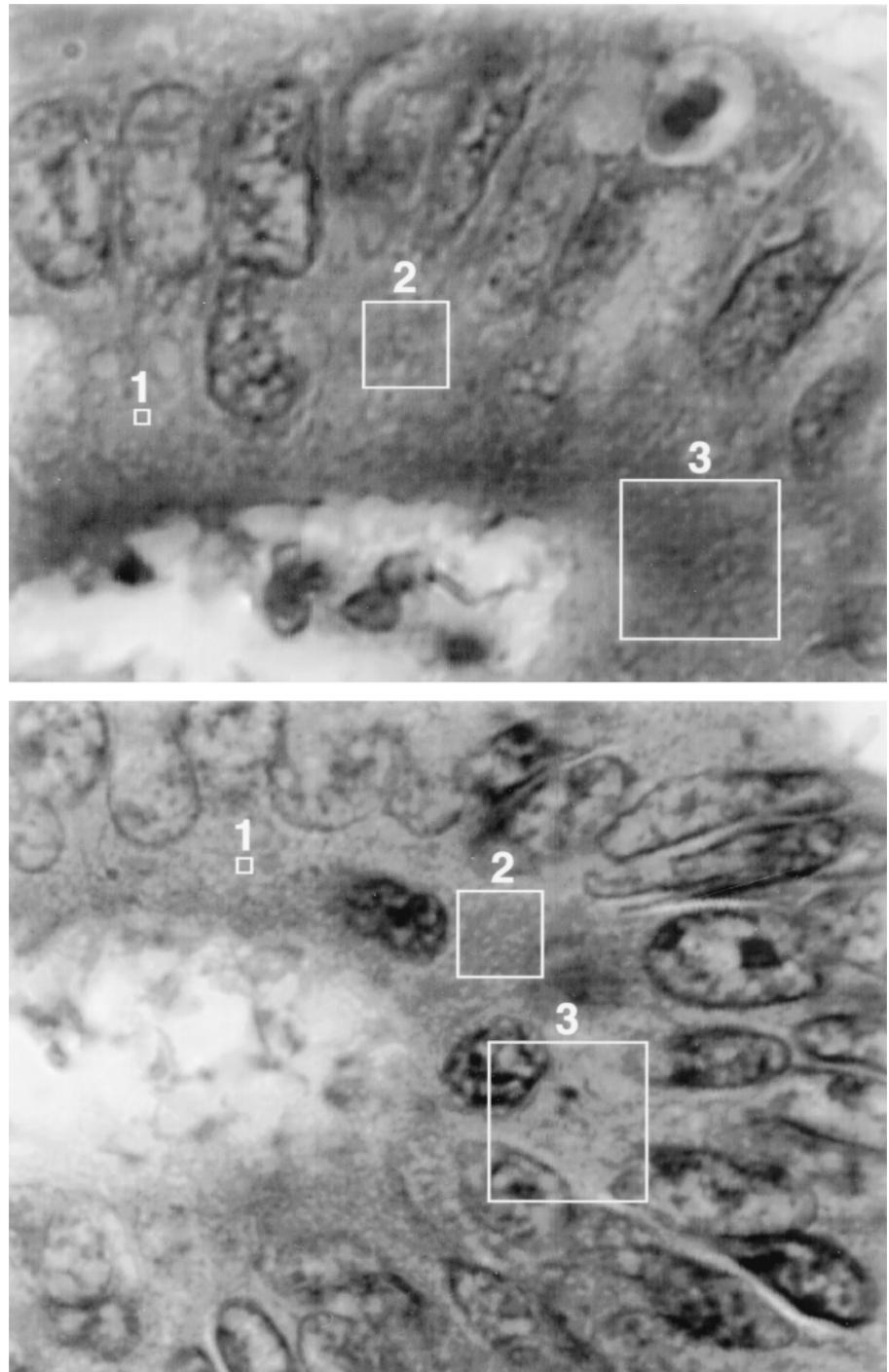
Figure 1 Immunohistochemistry performed using an antibody to GRPR on four different human colon cancers of various degrees of differentiation. RGB color images were obtained using a 3380×2700 pixel Microlumina digital camera after immunohistochemistry was performed on a well-differentiated cancer (A), and on cancers that were moderately differentiated (B), moderately poorly differentiated (C) and poorly differentiated (D). Insets show control tissues not exposed to antibody. All specimens were processed at the same time, as described in Materials and Methods.

It is well appreciated that increasing exposure to DAB increases the amount of nonspecific staining of tissues. Therefore, we next evaluated the effect of varying the time of DAB exposure. Sequential sections were evaluated after exposure to DAB for 15 sec to 10 min (Figure 3). In all instances, control tissues were processed simultaneously and treated identically to the experimental tissue, except that they were not exposed to primary antibody. For short DAB exposure times (i.e., <2 min), the calculated energy increased logarithmically but then plateaued thereafter (Figure 3). Therefore, for DAB exposure times of more than 2 min, the amount of cytoplasmic chromogen remained constant as a function of tumor differentiation. This result is best explained as due to the fact that once the antigenic sites of interest have reacted with DAB, all

subsequent nonspecific increases in “brown” due to DAB equally affect the control and experimental tissues. This result also supports our exposure of tissues to DAB for 2 min, and indicates that longer times of exposure do not significantly alter experimental results.

We next determined if our results could be influenced by the time tissues were exposed to counterstain. We treated consecutive experimental and control sections of well, moderately, and poorly differentiated tumors with DAB for 2 min and then exposed them to Gill’s hematoxylin for various lengths of time. Because the control tissue was exposed to counterstain for the same amount of time as the experimental tissue, no significant differences in chromogen quantity were detected (Table 2). Therefore, chromogen quantity, as

Figure 2 Relative sizes of different windows used to determine energy for Q-IHC. The actual sizes of a 10×10 pixel window (Box 1), a 100×100 pixel window (Box 2), and a 200×200 pixel window (Box 3) are shown in a well-differentiated colon cancer (top) and in a moderately poorly differentiated colon cancer (bottom). The 200×200 pixel window cannot be placed over a homogeneous-appearing region in the less well-differentiated cancer, therefore requiring that smaller windows be used for analysis.



determined by our algorithm, is independent of the time of exposure to counterstain.

Finally, we studied whether our algorithm suffered from day-to-day variability. We determined chromogen quantity in sequential sections of well, moderately, and poorly differentiated colon cancers on 4 consecutive days. The average amount of chromogen for all 4 days was 267.8 ± 4.7 eu/pixel for well-differ-

entiated cancers, 171.8 ± 8.4 eu/pixel for moderately differentiated cancers, and 4.1 ± 1.9 eu/pixel for poorly differentiated cancers. Coefficients of variation ranged from 0.1 to 0.3 for all observations, indicating negligible variation in results across time (Table 2). These data therefore indicate that this algorithm provides consistent results independently of DAB exposure time (for times ≥ 2 min), duration of exposure to

Table 1 Amount of cytoplasmic GRPR chromogen present in human colon cancers of various degrees of differentiation as a function of the window size used for analysis^a

Differentiation status	10 × 10 pixels (eu/pix)	100 × 100 pixels (eu/pix)	200 × 200 pixels (eu/pix)
Well	267.1 ± 15.7 (0.091)	256.4 ± 8.9 (0.054)	236.7 ± 2.2 (0.014)
Moderate	167.3 ± 7.5 (0.070)	182.1 ± 1.4 (0.012)	198.7 ± 2.8 (0.021)
Moderately poor	55.6 ± 7.9 (0.219)	55.2 ± 1.3 (0.036)	61.1 ± 2.2 (0.057)
Poor	5.1 ± 1.1 (0.333)	5.1 ± 0.5 (0.158)	32.2 ± 5.4 (0.261)

^aAmount of GRPR-specific chromogen expressed in terms of energy units/pixel (eu/pix), with the data expressed as the means ± SEM of three separate observations. Coefficients of variation between the observations are shown in parentheses. Chromogen quantity was determined by subtracting the control image from the experimental image as described in Materials and Methods, using window sizes shown in Figure 2.

counterstain, and without significant day-to-day variability.

Discussion

Currently available algorithms for Q-IHC are best suited to counting pixel number (Kuyatt et al. 1993; Kohlberger et al. 1996) or to measuring area (Goldlust et al. 1996). Although these approaches are adequate for comparative assessments, they do not allow quantification of the absolute amount of chromogen

present in an image or a portion of an image. Because pixels have dimension, algorithms based on pixel counting (Lehr et al. 1997,1999; Ruifrok 1997) generate information concerning area. Therefore, the area occupied by any particular color range can be specified and compared in a relative manner, but it is not possible to determine the absolute amount of chromogen present. Furthermore, algorithms based on color thresholding followed by an enumeration of the number of pixels present within a color/brightness range employ the *a priori* reasoning that the only information worth evaluating exists within a predefined spectral range.

These limitations can be appreciated by briefly reviewing how color images are represented digitally. Each pixel, $x_{1,2}$, contained within an image is represented by separate grayscale values for each of the three primary colors. Thus, any image can be graphically depicted in red, green, or blue such that $f_i(x)$ is the intensity of color "i" represented by the number of pixels with gray level x_i (Figure 4). In essence, this is the representation provided in Photoshop's histogram function, and shows that any specific color is but a linear combination of the three primary colors. For the

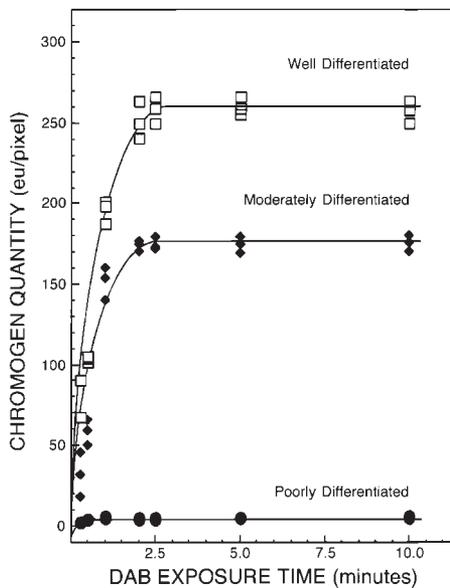


Figure 3 Demonstration that chromogen quantity does not significantly change with increased times of exposure to DAB. Sequential sections of three separate human colon cancers containing well, moderately, and poorly differentiated tumor regions were treated with DAB for the indicated times. Control and experimental tissues were processed similarly except that the former were not treated with primary antibody. Q-IHC was performed using a 100 × 100 pixel window on three separate cytoplasmic regions and values averaged. Data points represent the means per tissue specimen, with each tumor specimen shown separately. Independent data points for poorly differentiated tissues cannot be distinguished because of overlapping values.

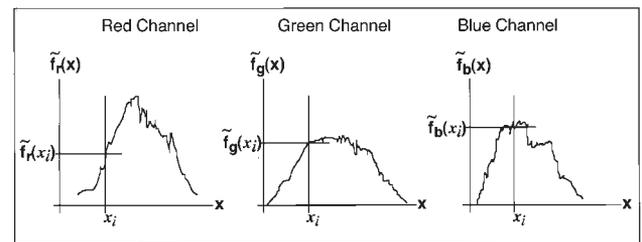


Figure 4 Graphic representation of how any specific color can be represented by combining the brightness, "x," for each of the primary colors, red, green, and blue, as shown using a histogram analysis. The intensity of color, "i," is represented by the number of pixels of brightness (i.e., gray level) x_i . In 24-bit color, x ranges from 0–255 (i.e., 2^8 levels) for each primary color. The color generated for any pixel $x_{1,2}$, therefore, is determined as shown in equation (1). In contrast, the cumulative signal strength is represented by equation (3), as described in the Discussion.

Table 2 Chromogen quantity is independent of the amount of counterstain (A) or the day on which immunohistochemistry is performed (B)^a

A.					
	Counterstain time (min)				
Differentiation status	0.5	1.0	2.0	4.0	CV
Well	250.7 ± 4.9	261.3 ± 8.9	289.5 ± 2.5	277.4 ± 4.4	0.05
Moderate	159.1 ± 2.4	155.7 ± 2.8	152.2 ± 2.8	148.8 ± 4.2	0.07
Poor	3.6 ± 0.2	4.4 ± 0.8	3.7 ± 1.0	2.9 ± 1.4	0.20
B.					
	Day of assay				
Differentiation status	Day 1	Day 2	Day 3	Day 4	CV
Well	269.7 ± 3.2	263.6 ± 11.2	280.1 ± 5.3	257.9 ± 9.4	0.04
Moderate	174.2 ± 5.8	154.4 ± 8.2	164.7 ± 6.9	193.8 ± 7.1	0.10
Poor	4.2 ± 0.6	4.6 ± 0.4	3.9 ± 1.1	3.7 ± 0.3	0.09

^aConsecutive colon cancer sections (5 μm thick) containing all three differentiation types were counterstained with Gill's hematoxylin for the indicated amount of time (A) or for 1 min on 4 consecutive but separate days (B). Cytoplasmic chromogen (eu/pixel) was determined using three 100 × 100 pixel windows, with data shown as means ± SEM. CV, coefficient of variation between results per tissue differentiation type.

DAB-generated “brown” in our immunohistochemistry, therefore, any pixel $x_{1,2}$ can be represented by:

Equation (1)

$$f_{\text{“BROWN”}}(x) = \alpha_1 f_{\text{RED}}(x) + \alpha_2 f_{\text{GREEN}}(x) + \alpha_3 f_{\text{BLUE}}(x)$$

for the unique predetermined set of coefficients α_i , $i=1,2,3$, corresponding to the color “brown.” As an aside, it should be observed that these coefficients are used to characterize the perceived “brown” component of the image as viewed by the user from the digital file. The image generated by the digital file, however, displays the three primary colors simultaneously and depicts the entire visual spectrum (i.e., the visual system enables us to perceive all possible colors simultaneously).

For quantitative purposes, one could attempt to isolate color using equation (1). However, such an approach has three significant limitations. First, the α coefficients need to be determined. These coefficients vary for each different wavelength studied and must be determined experimentally for each tissue section evaluated. Second, the range of wavelengths involved in generating the color “brown” generally is not known to the viewer and needs to be determined, especially to allow determination of the α coefficients. Third, this approach would be useful only for studies using systems generating chromogen of identical “brown” color. In other words, such an algorithm would not be transferable to techniques using other color detection systems.

These limitations can be circumvented by assessing all the changes in the experimental image corresponding to the additive contributions of chromogen to the control image. This is manifested by additive changes in the entire color spectrum, or to the red, green, and blue representations encoded within the image's digital file. When DAB is used these changes generally appear in the “brown” spectrum, but our technique applies equally to any color-related change. Specifically,

we calculate the cumulative strength, or energy, of the control image and subtract this value from that of the experimental image. For an image represented as a continuous variable as shown in Figure 4, energy is:

Equation (2)

$$E = \left[\int_{-\infty}^{+\infty} \int_{-\infty}^{+\infty} [f_{\text{RED}}(x_1, x_2)]^2 dx_1 dx_2 \right]^{1/2} + \left[\int_{-\infty}^{+\infty} \int_{-\infty}^{+\infty} [f_{\text{GREEN}}(x_1, x_2)]^2 dx_1 dx_2 \right]^{1/2} + \left[\int_{-\infty}^{+\infty} \int_{-\infty}^{+\infty} [f_{\text{BLUE}}(x_1, x_2)]^2 dx_1 dx_2 \right]^{1/2}$$

where x_1 and x_2 identify the specific position of the data encoded within the matrix representing the image data file (Jain 1989). Thus, energy defined mathematically refers to the notion of “norm” or cumulative signal strength (Jain 1989). This is analogous to but completely different from its definition in physics, where energy conveys the notion of the cumulative amount of work performed over time.

In 24-bit color, red, green, and blue are represented as discrete variables, with each channel limited to integrating brightness values between 0 and 255. Thus, equation (2) becomes:

Equation (3)

$$E = \left[\sum_{n_1=1}^{N_1} \sum_{n_2=1}^{N_2} [f_{\text{RED}}(n_1, n_2)]^2 \right]^{1/2} + \left[\sum_{n_1=1}^{N_1} \sum_{n_2=1}^{N_2} [f_{\text{GREEN}}(n_1, n_2)]^2 \right]^{1/2} + \left[\sum_{n_1=1}^{N_1} \sum_{n_2=1}^{N_2} [f_{\text{BLUE}}(n_1, n_2)]^2 \right]^{1/2}$$

for an image matrix file of dimensions N_1 by N_2 pixels (and where n_1 and n_2 still identify the specific position any particular value contained within the image file matrix). To calculate the chromogen-specific energy, we subtract the energy of the region contained in the control image from the homologous region of the experimental image such that

Equation (4)

$$E_{\text{chromogen}} = (E_{\text{experiment}}) - (E_{\text{control}})$$

In this report we show that Q-IHC performed using this algorithm reveals a ~ 50 -fold difference in GRPR expression between well-differentiated and poorly differentiated colon cancers. This range is well beyond that which otherwise could be accomplished by individual investigators studying analog images using a 0–4 grading scale. The precision, as shown by the small coefficients of variation as well as the numerical range of the data obtained using this algorithm, allows chromogen amounts to be subjected to meaningful statistical analysis. In this study we evaluated four or five separate tumors of varying histology per experiment. Overall, we found that the amount of GRPR-induced chromogen was significantly higher in well-differentiated cancers (266.8 ± 5.7) as compared to moderately (171.8 ± 7.8) or poorly differentiated tumors (4.1 ± 1.1) ($p < 0.01$, ANOVA). Therefore, Q-IHC permits significant differences between various immunohistochemical images to be elucidated with a hitherto unachievable degree of precision that is independent of the time of exposure to DAB (Figure 3) or counterstain (Table 2), and is devoid of significant day-to-day variability (Table 2).

Q-IHC as performed in our study also reduces potential observer bias when specific cell regions are being evaluated. Nonhomogeneous tissue specimens (i.e., Figure 2C) are difficult to evaluate because the actual amount of cytoplasmic chromogen present is small relative to distracting features such as vacuoles and nuclei. By determining the energy resident in a number of tissue regions containing only cytoplasm, the precise amount of chromogen specific to that region can be calculated. Although we did not specifically evaluate this process in this study, chromogen present in the nucleus distinct from that observed in the cytoplasm also can be quantified by using the appropriately sized window. Finally, the algorithm described is not restricted to evaluation of DAB-based immunohistochemistry. Because it is the image file itself that is subject to analysis, the algorithm described herein can be used to quantify chromogen regardless of the type of secondary antibody detection system.

It might appear that the use of an RGB camera is unnecessary and that a simple monochrome camera could suffice. Although a monochrome camera might

be adequate in some cases, it would not perform well in all circumstances. The major limitation to the use of a monochrome camera is the fact that, unlike RGB cameras, the color space is represented equivalently by either the RGB color space or the IUUV color space. The RGB color space is used to denote the decomposition of all images into the three primary colors: red, green, and blue, whereas the IUUV color space provides a decomposition of all images into the illuminance (I) and two chrominances (U and V). In contrast, the monochrome camera captures only the illuminance component of the image and the information for chrominance (U and V) is completely ignored. Although our algorithm could be used to determine the amount of chromogen present in the image specimen based on the energy differences present in the illuminance, this approach would lead to a high error rate because the differences contained by the chrominance images would be lost.

Even though our algorithm is best served using a more expensive RGB digital camera, expensive software is not necessary. Photoshop is commonly included in the purchase price of desktop scanners and otherwise can be purchased by students for $\sim \$100$. Similarly, Matlab can be obtained by students for the same price, providing a version that can assess matrices of up to 128×128 pixels. Larger images can be studied using the professional version of Matlab, which can be obtained for $\sim \$500$ (academic pricing). Therefore true Q-IHC, which allows the absolute amount of chromogen present to be determined, is possible for all individuals operating in a digital imaging environment.

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