Automatic Nonsubjective Estimation of Antigen Content Visualized by Immunohistochemistry Using Color Deconvolution

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Abstract: We describe a method for the automatic, nonsubjective estimation of 3,3′ diaminobenzidine (DAB) in digital images obtained from routine central nervous system immunohistochemistry using freely available, platform-independent public domain image processing software. This technique estimates the amount of antigen visualized but does not measure antigen content directly. Combined with whole brain section high-resolution scanning, a "virtual dissection" (extracting the region of interest) makes it possible to estimate relative antigen content in either subcellular structures, specific brain regions, or in whole tissue sections at magnifications up to 40 ×. The digital image is processed using Ruifrok and Johnston's color deconvolution method to separate the brown DAB chromogen from the hematoxylin counterstain on a microscope slide. A monochrome image representing the DAB content is then subjected to frequency analysis using NIH-ImageJ and a weighting calculation to estimate the amount of DAB (antigen) as a dimensionless index. The method described produces results that agree with enzyme-linked immunosorbent assays, and is automatic and nonsubjective. The method could easily be adapted to other types of tissue or cell cultures.

Key Words: antigen estimation, color deconvolution, immunohistochemistry, virtual microscopy, brain

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Modern immunohistochemical methods allow visualization of antigens in the context of structure but do not allow estimation of the amount of antigen present. Nonetheless differential staining of cytoplasm, cell nuclei, cellular organelles, for specific proteins or peptides is a potent technique for the study of normal and pathological states.

Immunohistochemistry uses specific interspecies antibodies to differentially stain tissue for antigens of choice. After application of a primary antibody, amplification can be effected with systems such as streptavidin-biotin coupled to 3,3′ diaminobenzidine (DAB), which is precipitated to a brown reaction product in the presence of hydrogen peroxide. This technique relies on the principle that greater antigen content leads to increased precipitation of DAB (or other chromophore). Analysis of immunohistochemical staining using DAB counterstained with hematoxylin is attractive as it can be combined with morphometric analysis of the chosen antigen in single brain regions or subregions that are normally inaccessible by conventional methods such as dissection.

Techniques for quantifying immunohistochemistry have been attempted by a number of other authors using chromophores with minimal overlapping absorption bands. Narrow-band filters can be used to selectively measure absorption at chromophore-specific wavelengths. However, there is generally still enough overlap in the absorption spectra of conventional stains to confound repeatable quantitation.[1]

The use of color transformation techniques based on red-green-blue (RGB) intensity from 3-channel cameras can, in principle, produce segmentation based on color. However, it is hardly ever the case that areas on a section are stained for 1 color only and RGB methods cannot separate the contributions of 2 or more stains. Even using transformations such as thresholding, underestimation of the relative contributions of each stain is difficult to avoid.

Ruifrok and Johnston[2] have developed a flexible mathematical method for separation of the color information in RGB images. Their technique can be applied to a number of image types, including sections stained using immunohistochemical methods. The method they describe treats the RGB information as a 3-dimensional space and colors are separated mathematically. This color deconvolution technique can separate combinations of 2 or 3 colors, provided those colors are sufficiently different in their red, green, or blue absorption characteristics. This deconvolution technique does not depend on thresholding

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nor is it limited by the possibility of overlapping absorption spectra. On the basis of an orthonormal transformation of the original RGB image given user-determined color vectors, color deconvolution can be used to determine staining densities in areas where multiple stains are colocalized.

As the RGB sensitivity of modern cameras and scanners is matched to the RGB sensitivity of the human visual system, almost every set of 3 colors that can be discriminated by the human eye can also be separated by this color deconvolution method.

The amount of chromophore on a section can be measured as the optical density at stain-specific wavelengths according to the Beer-Lambert law, with optical density proportional to the chromophore concentration. Although DAB cannot strictly obey the Beer-Lambert law as it scatters rather than absorbs light, it will be shown here that this is a theoretical rather than practical limitation to the method we describe.

We combine previously published techniques for color deconvolution with high-throughput, high-resolution digital microscopy or scanning with a high-resolution flat bed scanner (optical 4800×4800 dots per inch). To amplify the DAB signal and to eliminate the need to set thresholds subjectively, we perform a weighting calculation on the monochrome DAB image, multiplying the frequency by the intensity and expressing this as a percentage of the total number of pixels. Importantly, our method provides confirmation of qualitative assessment in a non-subjective manner. Furthermore, we demonstrate that it is possible to control for batch-to-batch slide staining variability. The software used to perform these analyses is platform independent, in the public domain, and freely available.

MATERIALS AND METHODS

The images used to develop and test this nonsubjective method of antigen estimation were obtained in the course of separate studies of intracerebral hemorrhage (ICH), which were approved by the Ethics Committees of the University of Adelaide and Institute of Medical and Veterinary Science. Experiments adhered to the National Health and Medical Research Council Code of Practice for Animal Research.

All experimental work was performed on male Sprague-Dawley rats (n = 45), weighing between 300 and 340 g, which were obtained at least 4 days before experiments, group housed and allowed access to food and water ad libitum. Sections of normal human brainstem were obtained from the South Australian Brain Bank and used as positive staining controls.

Intracerebral Microinjection of Collagenase and Thrombin

ICH was induced in rats using collagenase as previously described.[3] Briefly, anesthesia was induced with 5% isoflurane in a 30:70 mix of oxygen and nitrogen. Animals were then placed on a nose cone and transferred to a stereotactic frame (Kopf instruments) on a heat pad. Collagenase (Sigma C0773) 0.2 U in 2 μL normal saline or equal volume of vehicle was injected into the striatum through a burr hole at stereotaxic coordinates: anteroposterior: 0.7 mm, mediolateral: − 3.0 mm, and dorsoventral: 6.0 mm relative to Bregma, at a rate of 0.5 μL/min. The needle was left in place for 5 minutes and then withdrawn slowly. The burr hole was sealed with bone wax and the scalp closed after irrigation with bupivacaine.

Intracerebral injection of thrombin (n = 10) was performed in an identical fashion to injection of collagenase, except that 5 U of rat thrombin (Sigma T5772), in 5 μL oxygen-free normal saline was injected at a rate of 1 μL per minute.

Rats for histological processing (n = 30) were reanesthetized with barbiturate 5 or 24 hours postinjury and transcardially perfuse-fixed with 10% neutral buffered formalin. Brains were removed and postfixed for a minimum of 36 h in neutral buffered formalin before processing. Rats for enzyme-linked immunosorbent assay (ELISA) (n = 15) were decapitated under isoflurane anesthesia at 5 or 24 hours postinjection. Brains were immediately removed, dissected, snap-frozen in liquid nitrogen and stored at − 80°C for later analysis.

Histological Processing

Standard techniques were used to prepare sections for digital microscopy. Fixed brains were sectioned into 2 mm coronal slices using a Kopf brain blocker, placed in individual cassettes and processed to paraffin wax using a VIP automated tissue processor. The brain slice containing the striatum was cut into 5 μm sections, deparaffinized, and processed for immunohistochemistry. In brief, sections were dewaxed on a hot air blower and in xylene, then dehydrated in alcohol before being quenched for endogenous peroxidase activity with 0.5% hydrogen peroxide in methanol for 30 m followed by a 0.1 M phosphate-buffered saline (pH 7.4; PBS). Sections to be immunostained for substance P were antigen retrieved using an ethylene diamine tetra-acetate buffer (pH 8.0), whereas those sections immunostained for albumin did not require antigen retrieval. Nonspecific binding was blocked with 3% normal horse serum. Polyclonal antibodies to substance P (goat anti-substance P 1:2000, Santa Cruz sc-9758), and albumin (goat anti-albumin 1:20000, Cappel 0113-0341) were applied overnight at room temperature. The following day, sections were washed in PBS and a biotinylated anti-goat IgG was applied (1:250, Vector) for 30 minutes. This was followed by another PBS wash and incubation with a streptavidin peroxidase conjugate (1:1000, Pierce) for 1 hour. The immune complex was visualized by the precipitation of DAB (Sigma, D-5637) in the presence of hydrogen peroxide. Sections were washed again, counterstained with hematoxylin, dehydrated, cleared, and mounted.

Enzyme-linked Immunosorbent Assay

ELISA for substance P was performed on tissue obtained from the ventral half of the striatum (a 4 mm thick slice centered on the injection site) and the overlying

cortical and subcortical structures. Immediately after decapitation, the tissue was weighed, dissected, frozen in liquid nitrogen, and stored at −80°C. For assay, tissue was transferred to a glass Dounce type homogenizer and a 10:1 ratio of homogenization buffer (100 mM TRIS pH 7.4 in 0.9% saline; TBS) was added. The tissue was homogenized with 10 strokes using the loose pestle followed by 10 strokes using the snug pestle. The homogenizer was kept cold in an ice bucket. The homogenates were transferred to centrifuge tubes, rinsing the homogenizer 3 times with TBS then vortexed on ice every 5 minutes for 20 minutes before being centrifuged at 8500 rpm for 15 minutes. The supernatant was retained and refrozen.

A 50 μL aliquot of supernatant was taken for estimating protein content against a standard curve derived from serial dilutions of bovine serum albumin (Sigma A2153). Five microliters of supernatant or bovine serum albumin was added to recommended amounts of Biorad protein assay reagents (500 to 0113, −0114 and −0115). Three wells were performed for each specimen and readings averaged. Absorbance was read at 620 nm.

For ELISA, aliquots of supernatant were diluted with TBS to a protein concentration of 400 ng per 100 μL. For the ELISA assay, a standardized amount of protein is loaded into each well (400 ng/100 μL TBS). In this way, changes in antigen content cannot be attributed to differing content of protein.

Triplicate samples were added to a 96-well plate (Nunc, F96 Maxisorp). Blank wells were included as controls. Specimens were allowed to coat the wells overnight at 4°C before being tipped out and wells blocked with 0.5% gelatin for 1 hour. After rinsing 3 times with TBS 100 μL SP antibody (Chemicon, 1:1000, AB1566) was added and incubated at 37°C for 1 hour. Three further rinses were performed and 100 μL anti-rabbit horse radish peroxidase added (Molecular and Life Sciences Biobar, 1:500) and incubated as above. Specimens were drained and rinsed 4 times in TBS. The liquid substrate system 3,3,5,5-tetramethylbenzidine was added (100 μL/well) for 150 seconds and the reaction stopped with 50 μL of 0.5 M H_2SO_4. Absorbance was read at 450 nm with an Ascent Multiskan Plate reader.

Automatic, Nonsubjective Estimation of DAB

Digital images of whole coronal sections that included the injection site were acquired using a Nanozoomer Digital Pathology Scanner at a magnification of 10× or 40× (NDP Scan U10074-01, Hamamatsu Photonics K.K., Japan). For whole coronal images, scanning with a high-resolution flat bed scanner (optical 4800 × 4800 dots per inch) also gives an image suitable for estimating DAB-coupled antigen in a whole coronal section.

Slides were viewed with the associated proprietary viewing software and either whole coronal sections (albumin immunostaining) or peri-hematomal microregions (substance P immunostaining) were exported as jpeg2000 image files (the jpeg2000 specification provides for a lossless image format). Other defined microregions such as dentate gyrus or substantia nigra can be "virtually dissected" (extracting the "region of interest") using this method. For whole coronal images, the background (the canvas) was selected using the "magic wand tool" in Adobe Photoshop (allows selection of an area of an image based on its color) and set to RGB 255, 255, 255 (white). The exact threshold for the magic wand was selected empirically for each section and its accuracy is self-evident to the operator and easily adjusted.

Uneven illuminated background was removed using the "rolling ball" method.[4] A "rolling ball" of a predetermined radius is used to subtract background and remove large spatial variations of the background intensity. The radius of the "rolling ball" should initially be set to at least the size of the largest object that is not part of the background. We find that a radius of 50 pixels produces reliable color deconvolution results for routine use in our laboratory.

Hematoxylin (for visualization of histology) and DAB (for locating the antigen of interest) was digitally separated using Ruifrok and Johnston's[2] color deconvolution method implemented as an NIH-ImageJ macro. Code to implement this algorithm was obtained from Landini.[5] Landini's ImageJ plugin processes an RGB image and creates three 8-bit monochrome images. The first 2 represent hematoxylin (color 1) and DAB (color 2), and the third image represents the complementary of the first 2 colors (color 3, green). If the third image is not white or nearly white, then either the color vectors or the background color correction have not been correctly determined. We found the vectors provided with the macro sufficiently accurate.

The deconvolved DAB image was subjected to histogram analysis using NIH-ImageJ. The histogram list was then imported into a Microsoft EXCEL spreadsheet. This histogram list provides information on the number of pixels (*histogramvaluecount* in the NIH-ImageJ histogram list) at each pixel intensity (0 to 255, *histogramvalue* in the NIH-ImageJ histogram list), where 0 represents a pixel of darkest intensity and 255 represents a white pixel. To estimate the amount of DAB in a section, each pixel intensity was multiplied by the number of pixels at that intensity (0 to 255) and then summed.

1.

$$\mathrm{DAB} = \sum (\mathit{histogramvalue} \times \mathit{histogramvaluecount})$$

However as the darkest pixels, which represent positive staining, are worth 0, this equation is counter-intuitive as zero multiplied by its *histogramvaluecount* equals zero. We therefore inverted pixel intensity so that darkest pixel intensity is now worth 255 and the white pixels are worth 0; that is $255 - \mathit{histogramvalue}$. This improves signal to noise ratio and obviates the need to manually (and subjectively) adjust image thresholds. This weighted DAB (DABwt) value is dimensionless and is simply an index of the amount of DAB represented in an image.

2.

$$\mathrm{DABwt} = \sum [(255 - \mathit{histogramvalue}) \times \mathit{histogramvaluecount}]$$

To express this as a percentage of DABwt in the image, it needs to be divided by the "maximum theoretical DAB" then multiplied by 100. "Maximum theoretical DAB" is equivalent to every pixel in the image being maximally "brown" (value of 255). Accordingly, total number of pixels in the DAB monochrome image were summed and then multiplied by 255. However pixel counts of pixel intensities of 2 or less (white) were discarded as these represent empty blood vessels, ventricles, or areas outside the brain. Thus, "maximum theoretical DAB" was calculated by summing of the pixel counts (*histogramvaluecounts*) for pixel intensities of 3 to 255, then multiplying this number by 255.

This DABwt% was calculated using a Microsoft EXCEL spreadsheet with the following equation.

3.

$$\text{DABwt \%} = \left(\frac{\text{DABwt}}{\sum(histogramvaluecounts) \times ,255}\right) \times 100$$

Therefore, DABwt% values obtained represent an estimate of the amount of DAB (and thus antigen) on the original tissue section. The DABwt% can then be compared with different sections (images) and the results subjected to statistical analysis.

Directory spanning code was wrapped around the deconvolution and "rolling ball" procedures. A Microsoft VBA script was written to perform the weighting calculation and summarize the data in a single Microsoft EXCEL spreadsheet. Image files are named according to an agreed convention and the data filtering function in Microsoft EXCEL is used to analyze the DABwt% value for the experimental group being analyzed. Up to 600 images can be processed as a batch.

Method Validation and the Beer-Lambert Law

To determine whether DAB followed the Beer-Lambert Law for practical purposes and for the amounts of DAB used in our immunohistochemical methods, we stained control sections of human brainstem for substance P using serial dilutions of primary antibody (a minimum of 2 per dilution) to act as a surrogate measure of antigen dilutions. We also analyzed the relationship between DABwt% and time of DAB incubation. For further validation of the color deconvolution method, DABwt% following substance P immunostaining was compared with substance P estimation by ELISA for rats subjected to ICH and sacrificed at 5 and 24 hours.

Statistical Analysis

Statistical analyses were performed with GraphPad Prism v5.00 for Windows (GraphPad Software, San Diego, CA). Between-group comparisons of DABwt% were performed with 2-sided unpaired t-tests with a 0.05% significance level. Regression analysis was performed to assess the correlation of DABwt% with antibody dilution, as well as the correlation of DABwt% with incubation time. ELISA analysis was performed using a 1-way analysis of variance followed by Dunn's multiple comparison tests with a significance level set at 0.05%.

RESULTS

Qualitative assessment of DAB staining agreed with the automatic, nonsubjective estimation calculation of DABwt%. Furthermore, DABwt% from substance P immunostaining and substance P content in ELISA was comparable.

Correlation of DABwt% With Antibody Dilution, Incubation Time, and the Beer-Lambert Law

We calculated the amount of DAB (DAB weighted % of total, DABwt%) in a monochrome image extracted from the hematoxylin and DAB-stained sections using Ruifrok and Johnston's color deconvolution method. The monochrome image only contains information about the amount of DAB present thus eliminating the absorption spectrum. DAB cannot follow the Beer-Lambert Law, as it is not a true absorber of light, however the apparent content of DAB is, for practical purposes linear using our weighting calculation. There was a significant correlation between DABwt% and variable dilutions of primary antibody to substance P in human brainstem sections ($R^2 = 0.8866$; $P < 0.001$) (Fig. 1). Similar results were demonstrated with other antibodies in common use in our laboratory (data not shown). Similarly, variable DAB incubation periods showed a statistically significant correlation with DABwt% ($R^2 = 0.9698$; $P < 0.001$) (Fig. 1).

Comparison of Substance P Estimated by DABwt% and ELISA

Substance P as estimated by DABwt% was significantly elevated in the ipsilateral hemisphere compared to the contralateral hemisphere at both 5 and 24 hours after collagenase ICH, whereas vehicle controls had only a small nonsignificant increase in substance P content estimated by DABwt% in the ipsilateral hemisphere at 24 hours (Fig. 2). Comparable increases in substance P content were seen in the ipsilateral hemisphere after collagenase ICH using the semi-quantitation method of ELISA. These results suggest that DABwt% calculation provides a suitable method to estimate the amount of an antigen present in an immunohistochemically stained section.

DABwt% for Albumin Immunohistochemistry

After intrastriatal thrombin injection, albumin immunostaining clearly compared well with DABwt% (Fig. 3). Albumin immunoreactivity in the ipsilateral (right) striatum extended to the cortex along the needle path.

By 24 hours, albumin had spread throughout the entire ipsilateral hemisphere, into the contralateral hemisphere via the corpus callosum. Visual inspection of sections obtained 5 and 24 hours after intrastriatal injection of thrombin matched the calculated DABwt% value in the ipsilateral hemisphere of these animals. Indeed observers blinded to treatment were able to subjectively match up the DABwt% to each image (data not shown).

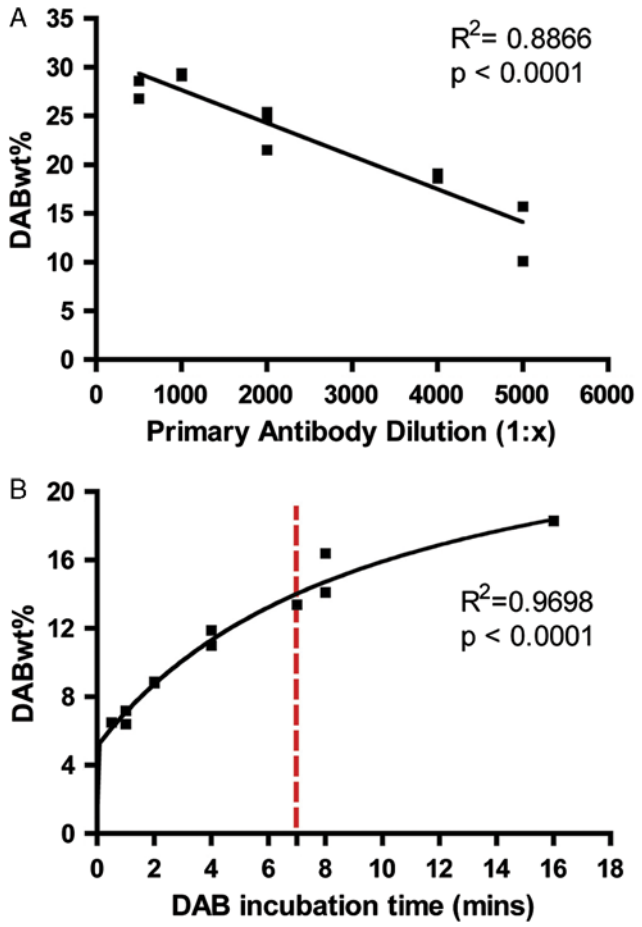

FIGURE 1. Correlation of DABwt% (percentage of weighted 3,3′ diaminobenzidine) with antibody dilution and incubation time of substance P antibody in sequential human brainstem sections. A, DABwt% with variable dilutions of primary antibody to substance P serving as a surrogate of protein content. B, DABwt% with variable DAB incubation periods. The dotted line represents the DAB incubation time for our standard immunohistochemical method. full color online

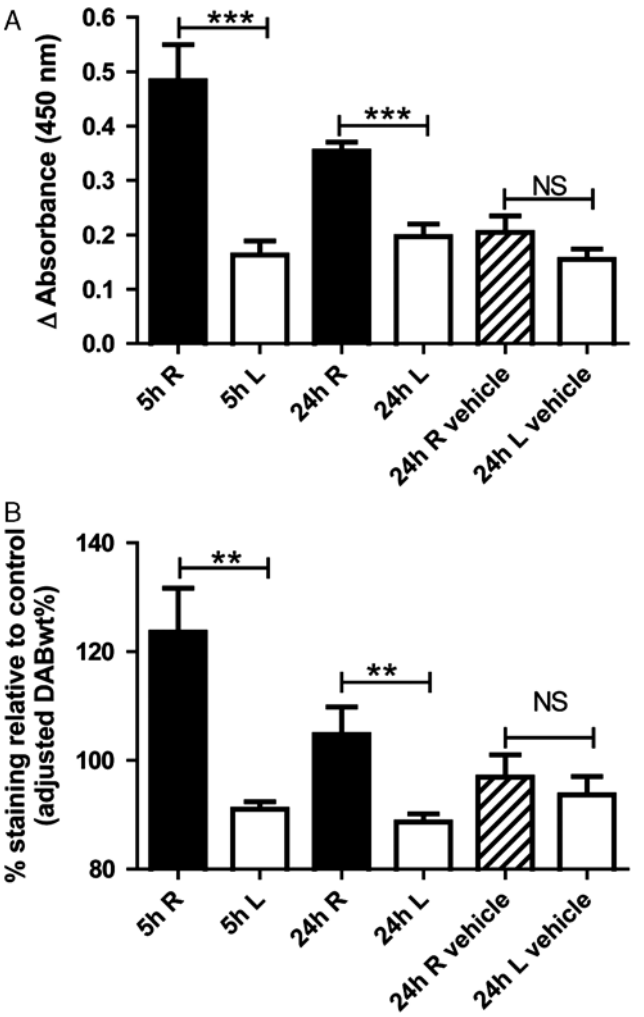

FIGURE 2. Comparison of substance P content as measured by enzyme-linked immunosorbent assay (ELISA) or estimated by DABwt% (percentage of weighted 3,3′ diaminobenzidine). A, Substance P as measured by ELISA at 5 and 24 hours after collagenase intracerebral hemorrhage, or vehicle control. B, Substance P as estimated by DABwt% adjusted to an internal control area at 5 and 24 hours after collagenase intracerebral hemorrhage, or vehicle control. $^{**}P < 0.01$, NS indicates not significant.

Selection of a Control Region for Staining Variability

Slight variations in immunohistochemical technique in different batches of staining can result in changes in DAB intensity, thus a control region may be used to "normalize" staining across batches. At high power magnification, staining in the interhemispheric leptomeninges did not differ in 1 U thrombin or vehicle treated animals if sections were processed within the same batch (Fig. 4). Therefore, this area was deemed a suitable control region for substance P staining in ICH injury.

Deliberate destruction of substance P by heating slides for 5 m with a hot air blower (normally used for dewaxing) diminished substance P staining intensity substantially. In contrast, dewaxing a consecutive section for only 1 m on the same hot air blower did not result in substantial losses of substance P immunoreactivity.

Overheating with the hot air blower also caused the interhemispheric leptomeninges to display faint substance P immunostaining, suggesting that interhemispheric leptomeninges staining can be affected by technique, but not injury.

Adjustment to a suitable control area is required for analysis of DABwt% and estimation of antigen content as even subtle variations in technique can lead to mistaken conclusions. This was particularly apparent for substance P immunostaining. When DABwt% is unadjusted for a control region, the analysis could suggest that thrombin injection caused substance P immunoreactivity to decrease bilaterally compared with vehicle controls (Fig. 5). However by using the interhemispheric leptomeninges as a control region, these errors were eliminated.

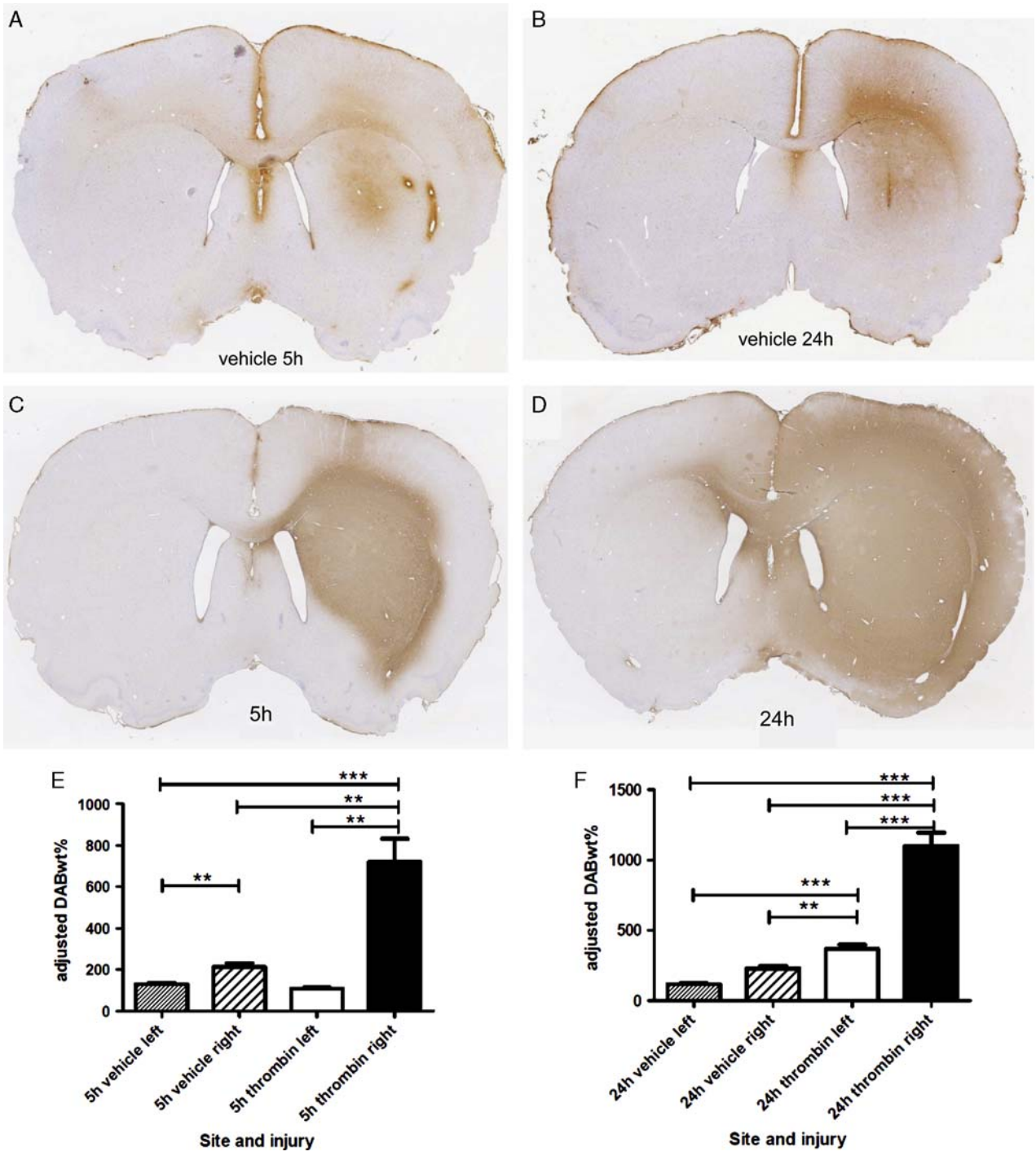

FIGURE 3. Comparison of albumin immunostaining postintrastriatal thrombin injection, as assessed visually and as estimated by DABwt% (percentage of weighted 3,3′ diaminobenzidine). A, Coronal section of rat striatum, albumin immunohistochemistry 5 h post-striatal injection of vehicle. B, Matched section at 24 h post-vehicle. C and D, Matched sections 5 and 24 h post intra-striatal injection of thrombin (1U). E and F, DABwt% analysis of sections obtained at 5 h (E) and 24 h (F). $^{**}P < 0.01\%$, NS indicates not significant.

DISCUSSION

Researchers spend considerable time preparing brain sections for immunohistochemistry as part of an investigation into some pathological state. At worst, analysis is subjective and descriptive, at best empirical grading systems are employed. Many groups resort to ELISA, polymerase chain reaction, or other quantitative methods in an attempt to objectively compare experimental

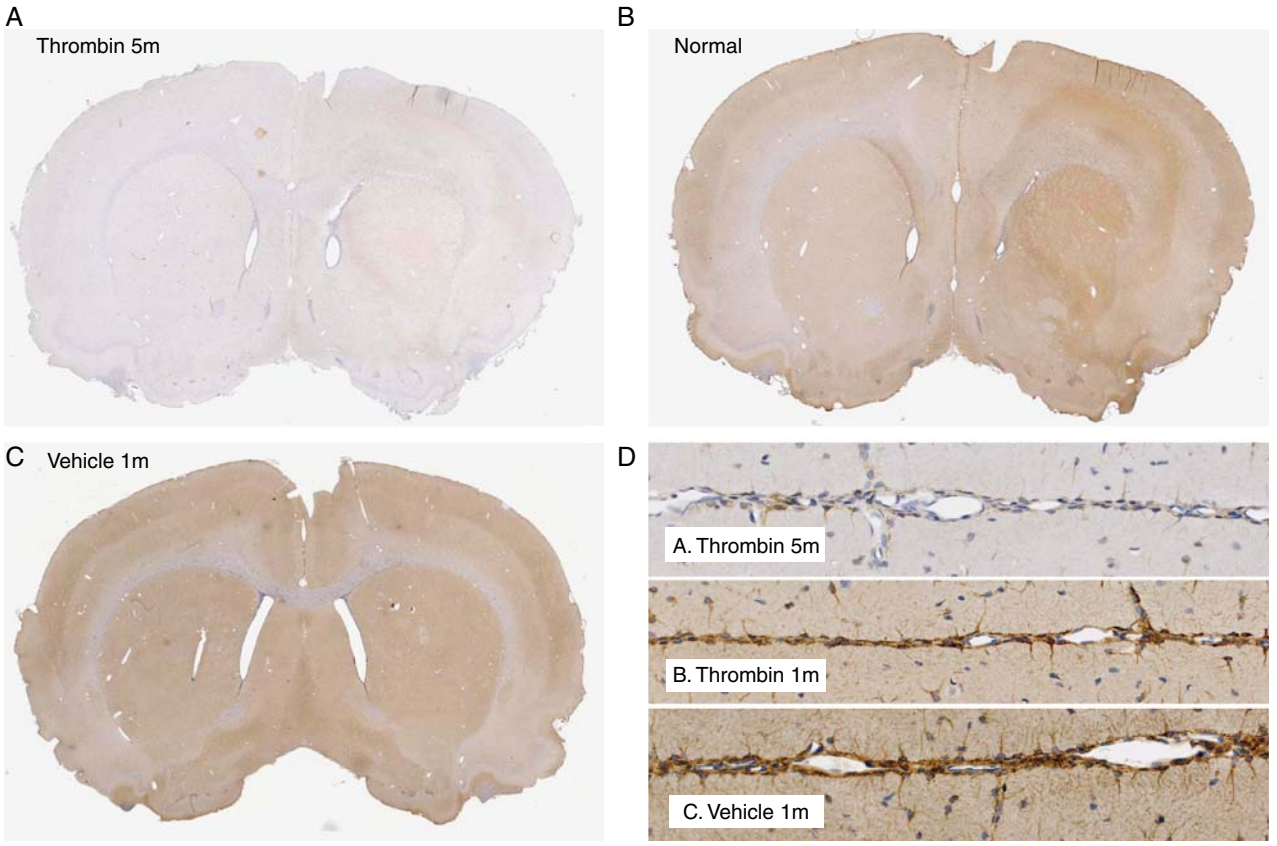

FIGURE 4. Variations in processing technique alter levels of substance P immunoreactivity in coronal sections of rat striatum at 24 hours after striatal injection of 1 U rat thrombin or vehicle. A, Dewaxing the section on a hot air blower for 5 m. B, Dewaxing a consecutive section for 1 m results in "normal" substance P immunoreactivity. C, Vehicle-treated control, dewaxed for 1 m. D, Variability of staining intensity at high power in the interhemispheric leptomeninges, where staining is affected by technique, but not injury. full color online

and control groups. Generally, a matching set of animals is required to generate tissue for these additional analyses, resulting in increased animal numbers and cost.

The method we describe here for calculation of the dimensionless DABwt% value provides a nonsubjective estimation of the amount of antigen present. Moreover, the virtual dissection (region of interest) makes possible the estimation of antigen content in areas of the brain that are inaccessible by other means. Calculation of the DABwt% is automatic (software program on a desktop computer) suggesting objectivity and reproducibility. According to Tadrous[6] the basic methodology of digital image analysis in histology follows 7 steps.

(a). Tissue preparation (perfuse fix, immersion fix)
(b). Acquire image (Nano-Zoomer, flat bed scanner, camera)
(c). Image preprocessing (JPEG data loss, RAW format)
(d). Image segmentation into regions of interest
(e). Postprocessing, (auto-leveling, blemish removal)
(f). Calculation of the metrics (DABwt%) using the segmented image
(g). Interpretation of the results.

It must be emphasized that both control and sample tissues have been subject to different degrees of "stress" at each of the 7 steps, any one of which can have a significant impact on antigen preservation and retrieval,[6] and therefore also on the calculated DABwt% value. At each step there are many subjective decisions to be made, although some can be frozen in program code. For example, we remove background using the "rolling ball" method. Parameters that may affect calculation of the DABwt% value (such as the radius of the "ball") are made inaccessible to inexperienced users. Furthermore we do not manually set any thresholds, either in code or by the end user. Multiplying the pixel intensity by the frequency automatically calculates a very strong signal for the darker (positive) areas on a section and a relatively weak signal for faint (negative) areas.

The Beer-Lambert law describes a linear relationship between the concentration of a compound and its optical density. As color deconvolution extracts the color of DAB only, for practical purposes there is no absorption spectrum. Furthermore, although antigen-antibody reactions are not stoichiometric, for the amounts of DAB precipitated by immunohistochemistry in our laboratory we find a near linear relationship between DABwt% and different primary antibody dilutions and incubation times for DAB.

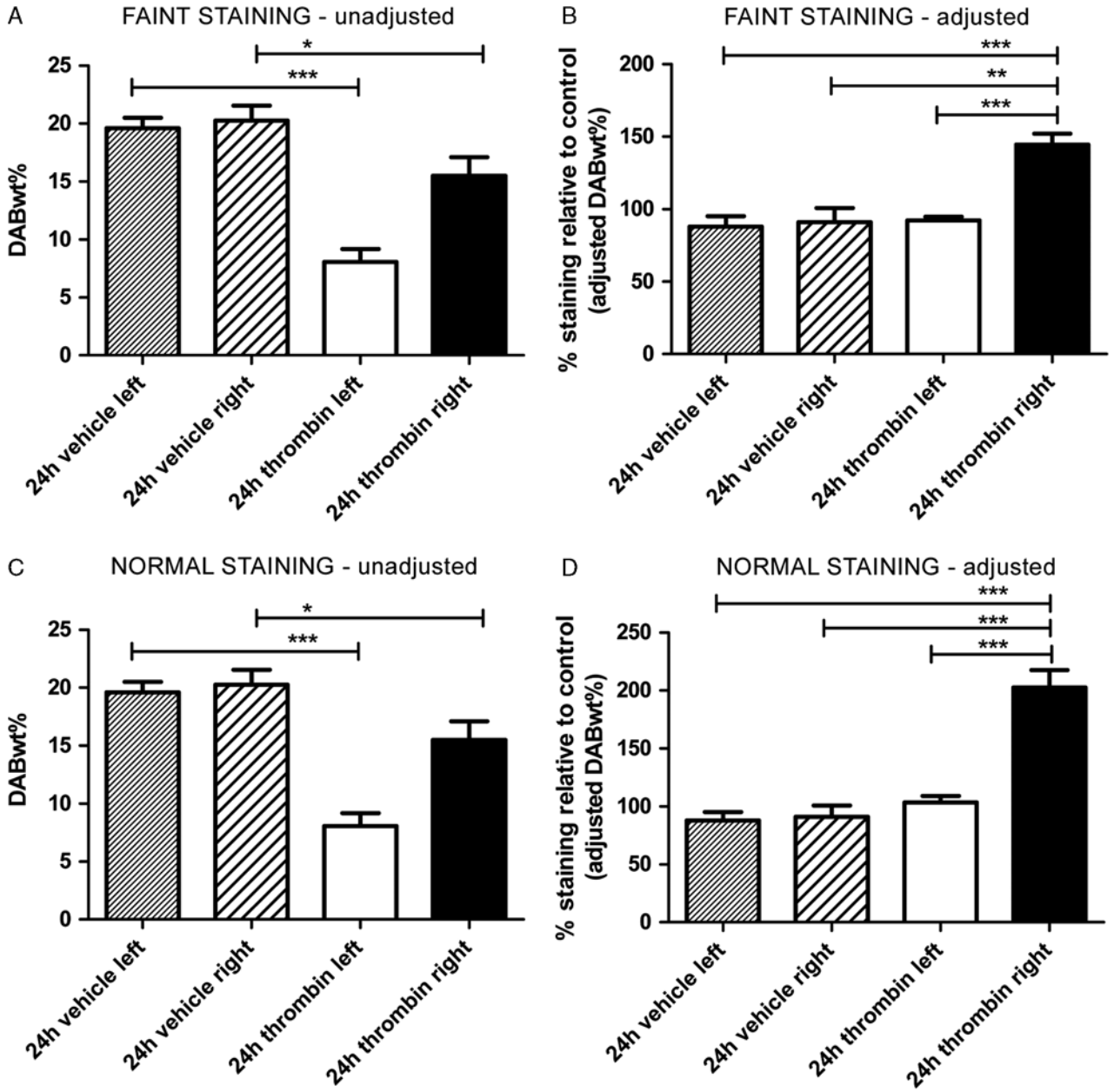

FIGURE 5. Adjustment of DABwt% (percentage of weighted 3,30 diaminobenzidine) to staining intensity in a control area interhemispheric leptomeninges, refer to figure 4). A and C, When unadjusted for a control area, DABwt% could suggest that thrombin injection causes either substance P immunoreactivity to decrease bilaterally compared with vehicle (A and C) or substance P immunoreactivity increases ipsilaterally after thrombin treatment or substance P is reduced contralaterally (C). B and D, adjusting for a control area prevents this error, as only the ipsilateral increase is apparent, which is in agreement with visual assessment of sections. $^{**}P < 0.01\%$, NS indicates not significant.

Further validation of this method was provided by comparison with the semi-quantitative method of ELISA. Here we were able to show that the amount of substance P antigen present as assessed by DABwt% was comparable to substance P content using an ELISA after an experimental model of ICH and in vehicle controls. Thus the method described here allows a reliable estimation of a particular antigen in both normal and pathological states.

Nonetheless a potential obstacle to the process described is that technical issues may cause variable "background" staining intensity or if the antigen is labile, our method may either not pick up a true change in antigen content, or falsely conclude that a change has occurred. This potential source of error can be mitigated if an internal control area can be identified; a region in which staining is present, but unaltered by injury. We found that for substance P immunostaining, the interhemipsheric

leptomeninges met this criteria, both after collagenase ICH and thrombin injections. The contralateral ventrolateral cortex served a similar purpose for albumin immunostaining. Thus, if the DABwt% obtained for each section is expressed as a percentage of internal control staining ("adjusted DABwt%"), valid conclusions are obtained for antigen content, even when processing and staining differences are quite extreme. If an internal control region cannot be identified (for instance in diffuse rather than focal brain injury), then tissue from a control section can be placed on the slide together with the section of interest to similarly normalize the content of antigen.

In conclusion, this paper brings together new imaging hardware with software and describes a technique for nonsubjective estimation of antigen content for brain immunohistochemistry for diffuse antigens such as substance P and albumin. The methodology described here is built on Ruifrok and Johnston's color deconvolution technique and uses software readily available to the research community. Although we describe our method using hematoxylin-counterstained DAB immunohistochemistry, it should be possible to generalize for other stains and tissues using the appropriate color deconvolution vectors. In this paper, we have shown that estimation of DAB precipitate using color deconvolution in combination with a calculation to weigh DAB content provides a novel approach to analyze immunohistochemistry. Crucially, this described method eliminates the need to set subjective thresholds.

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