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Quantification of histochemical staining by color deconvolution

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Running title: Color deconvolution and quantification

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Abstract

Objective: To develop a flexible method of separation and quantification of immunohistochemical staining by means of color image analysis.

Study Design: An algorithm was developed to deconvolve the color information acquired with RGB cameras, to calculate the contribution of each of the applied stains, based on the stain-specific RGB absorption. The algorithm was tested using different combinations of DAB, hematoxylin and eosin at different staining levels.

Results: Quantification of the different stains was not significantly influenced by the combination of multiple stains in a single sample. The color deconvolution algorithm resulted in comparable quantification independent of the stain combinations, as long as the histochemical procedures did not influence the amount of stain in the sample due to bleaching because of stain solubility, and saturation of staining was prevented.

Conclusion: The presented image analysis algorithm provides a robust and flexible method for objective immunohistochemical analysis of samples stained with up to three different stains, using a laboratory microscope and standard RGB camera setup, and the public domain program NIH image.

1. Introduction

Differential staining of cytoplasm, cell nuclei and other cell organelles, and specific proteins lies at the basis of pathology. One of the most common examples is the hematoxylin-eosin staining, with hematoxylin (blue) mainly staining the cell nuclei, and eosin (magenta-red) acting as a cytoplasmic stain. In addition to this, the ratio of hematoxylin and eosin staining of the cytoplasm also indicates basophilia or acidophilia of the cytoplasm. Another common example of differential staining is the use of immuno-histochemistry with e.g. horseradish peroxidase staining developed with 3,3' diaminobenzidine (DAB; brown). Immuno-histochemistry techniques can result in highly specific staining of numerous antigens. Slides stained with multiple stains can be used for the co-occurrence and co-localization of different markers, for example using a proliferation marker like Ki 67, together with a tumor-specific marker like the membrane-localized receptor Her2/Neu, diagnostic for specific breast tumors.

The goal of differential staining is to provide indicators of the distribution of the substance or structures to which the stain is specifically attached. The amount of stain attached or deposited will then determine the optical density at stain-specific wavelengths according to the Lambert-Beer law[1], with the optical density being proportional to the stain concentration. Although the dyes used for staining of the different structures or proteins are visualized to have different colors, they actually mostly have complex overlapping absorption spectra. To simultaneously examine the photometric and morphometric features of one or more structures, the relative contribution of each of the dyes to the resulting absorption spectrum, or the perceived colors, have to be separated. In regions with co-localization of the stains (e.g. staining with hematoxylin and DAB in Ki-67 positive nuclei), the quantification of each stain component cannot be determined at any single wavelength, because the optical density at any single wavelength is determined by the total absorption of the multiple stains.

To overcome the problem of separation of the contribution of multiple stains, three approaches have been used.

A. Use of individual components (stains) with non-overlapping absorption bands. Although some successes have been reported using this approach[2,3], unfortunately current cyto- and histo-chemistry still offer only a very limited number of such dyes.
B. Use of narrow-band filters to selectively measure absorption at a dye-specific wavelength. Although significant improvement of color separation has been made using this technique, it still suffers from the partial overlap in the absorption spectra of different dyes[4-7]. Matched filters are needed for every stain used, which may require considerable expense. Even with specifically matched filters, it is difficult to design a system so that each stain appears completely separated into a single color channel. Narrow band filtering changes the color representation, and reduces the color saturation in displays.
C. Use of color transformation techniques based on the red-green-blue (RGB) broad-band information from analog or digital three-channel cameras. Considerable success has been shown using color transformation techniques, either using the hue-

saturation-intensity (HSI) transformation[6,7] or similar transformations like hue-value-chroma (HVC)[8], or stain specific transformation[9,10]. A disadvantage of the color transformation techniques is that although the existing color transformations do result in segmentation based on color, they do not result in the separation of the contribution of two or more stains to the resulting color. Areas that are stained with two or more dyes are designated as one of the colors, depending on the relative contribution of the stains and threshold settings. This can result in considerable information loss when the above transformation techniques are used.

In histological and cytological staining it is hardly ever the case that areas are stained for one color only. This means that considerable information is lost when the above transformation techniques are used. Therefore we developed a novel color-deconvolution method that makes use of the broadband RGB information of standard analog or digital three-channel cameras. The method can be used for separation of practically every combination of two or three colors, provided that the colors are sufficiently different in their red, green or blue absorption characteristics. As the RGB sensitivity of cameras is matched to the RGB sensitivity of the human visual system, this means that virtually every set of three colors that can be seen as different colors by eye also can be separated by the color-deconvolution method.
The method is based on orthonormal transformation of the original RGB image, depending on user-determined color information about the three colors. The method provides the possibility to determine staining densities, even in areas where multiple stains are co-localized, making it possible not only to determine surface area and overall absorption in areas with a specific color, but also to determine densities and ratios of densities of stains in each area. After the color-deconvolution, images can be reconstructed for each stain separately, and be used for densitometry and texture analysis for each stain, using standard imaging methods.

2. Theory

A. Color representation.

In this discussion we assume a video microscopy system with a RGB camera, assuming that gray levels in each of the RGB channels are linear with brightness or transmission T, with T being I/I_0, with I_0 being the incident light and I the transmitted light. This assumption is reasonably accurate for CCD cameras with a gamma of 1.0.
Each pure stain will be characterized by a specific absorption factor c for the light in each of the three RGB channels. The detected intensities of light transmitted through a specimen and the amount (A) of stain with absorption factor c is described by Lambert-Beer's law[1]

$$I_C = I_{0,C} \exp(-Ac_C),$$

with $I_{0,C}$ the intensity of light entering the specimen, I_C the intensity of light detected after passing the specimen, and subscript c indicating the detection channel. As can be seen,

this means that the transmission of light, and therefore the gray-values of each channel, depend on concentration of stain in a non-linear way.
In the RGB model, the intensities I_R, I_G, and I_B are obtained by the camera for each pixel. Because the relative intensity in each of the channels depends on the concentration of stain in a non-linear way[11], the intensity values of the image cannot directly be used for separation and measurement of each of the stains. However, the optical density (OD) for each channel can be defined as

$$OD_C = -\log_{10}(I_C/I_{0,C}) = A * c_C .$$

As can be seen, the OD for each channel *is* linear with the concentration of absorbing material, and can therefore be used for separation of the contribution of multiple stains in a specimen.
Each pure stain will be characterized by a specific optical density for the light in each of the three RGB channels, which can be represented by a 3 by 1 OD vector describing the stain in the OD-converted RGB color space. For example, measurements of a sample stained with only hematoxylin resulted in OD values of 0.18, 0.20 and 0.08 for the R, G and B channels respectively

The length of the vector will be proportional to the amount of stain, while the relative values of the vector describe the actual OD for the detection channels. In the case of three channels, the color system can be described as a matrix of the form

$$\begin{bmatrix} p11 & p12 & p13 \\ p21 & p22 & p23 \\ p31 & p32 & p33 \end{bmatrix}$$

With every row representing a specific stain, and every column representing the optical density as detected by the red, green and blue channel for each stain. Stain-specific values for the OD in each of the three channels can be easily determined by measuring relative absorbtion for red, green and blue on slides stained with a single stain. An example of the OD matrix for the combination of hematoxylin, eosin and DAB is:

R	G	B	
0.18	0.20	0.08	Hematoxylin
0.01	0.13	0.01	Eosin
0.10	0.21	0.29	DAB

B. Color deconvolution.
To perform the separation of the stains, we have to do an ortho-normal transformation of the RGB information, to get independent information about each stain's contribution. The transformation has to be orthogonal to achieve independent information about each of the

stains; the transformation has to be normalized to achieve correct balancing of the absorbtion factor for each separate stain.
For normalization, we divide each OD vector by its total length,

$$\hat{p}_{11} = p_{11} / \sqrt{p_{11}^2 + p_{12}^2 + p_{13}^2}$$
$$\hat{p}_{31} = p_{31} / \sqrt{p_{31}^2 + p_{32}^2 + p_{33}^2}$$
$$\hat{p}_{21} = p_{21} / \sqrt{p_{21}^2 + p_{22}^2 + p_{23}^2}$$

etc.

resulting in a normalized OD matrix **M**:

$$\begin{bmatrix} \hat{p}11 & \hat{p}12 & \hat{p}13 \\ \hat{p}21 & \hat{p}22 & \hat{p}23 \\ \hat{p}31 & \hat{p}32 & \hat{p}33 \end{bmatrix}$$

The normalized OD matrix **M** for the above combination of hematoxylin, eosin and DAB is:

$$\begin{bmatrix} 0.65 & 0.70 & 0.29 \\ 0.07 & 0.99 & 0.11 \\ 0.27 & 0.57 & 0.78 \end{bmatrix}$$

If **C** is the 3 by 1 vector for amounts of the three stains at a particular pixel, then the vector of OD levels detected at that pixel is y = **CM**.

From the above it is clear that $\mathbf{C}=\mathbf{M}^{-1}[y]$. This means, that multiplication of the OD-image with the inverse of the OD matrix, which we define as the color-deconvolution matrix **D**, results in orthogonal representation of the stains forming the image;

$$\mathbf{C}=\mathbf{D}\,[y].$$

The color deconvolution matrix **D** corresponding to the above color matrix **M** for the hematoxylin, eosin and DAB matrix is:

$$\begin{bmatrix} 1.88 & -0.07 & -0.60 \\ -1.02 & 1.13 & -0.48 \\ -0.55 & -0.13 & 1.57 \end{bmatrix}$$

In this matrix, the diagonal elements are greater than unity, while the off-diagonal elements are negative. The above equation implies that the corrected OD level values for each stain are formed by subtracting a portion of the green OD and the blue OD from the enhanced red OD to obtain the hematoxylin OD, subtracting a portion of the red OD and the blue OD from the enhanced green OD to obtain the eosin OD, and subtracting a portion of the red OD and the green OD from the enhanced blue OD to obtain the DAB OD. In case the stains used would be pure red, green and blue stains, the above matrix would be the unity matrix.

Results of experiments using the above method are presented. Combinations of different staining intensities on the same slides are performed to test the influence of different levels of eosin counterstain on the quantification of DAB and hematoxylin.

3. Materials and Methods

Specimens

Sections from a breast tumor specimen were stained for Her2/neu expression with Her2/neu specific antibody (Oncogene Science, Cambridge, Massachusetts, USA), and DAB chromogen (Biogenics, Napa, California, U.S.A.). Counterstaining was performed with Mayer's Hematoxylin (Richard Allan, Kalamazoo, Michigan, USA) for 3-5 min and eosin (Polyscientific, Bay Shore, New York, U.S.A.) for different time periods under visual control. Sections from a lung tissue specimen were stained with Mayer's Hematoxylin (Richard Allan, Kalamazoo, Michigan, USA) for 3-5 min and eosin (Polyscientific) for different time periods under visual control. For determination of the effect of different eosin-staining levels on DAB and hematoxylin staining measurements, slides were de-stained in 95% alcohol or de-mineralized water, and re-stained with eosin for different time periods to reach a range of eosin staining intensities. Corresponding areas of slides were analyzed after each staining step, and stained area, OD and IOD were determined.

Image acquisition

A Leica DMLB (Leica Microsystems Inc. Deerfield, Illinois, USA) microscope was equipped with a Hamamatsu C5810 chilled 3-chip color CCD camera (Hamamatsu, Bridgewater, New Jersey, USA), interfaced with an IBM computer (International Business Machines Corporation, Armonk, New York, USA) equipped with a Matrox Meteor digitizer board (Matrox Electronic Systems Ltd., Dorval, Quebec, Canada). Light and camera settings were standardized, resulting in average background values of 20±5 (mean ± standard deviation; scale 0-255 from white to black) for the red, green and blue channels. Linearity of the image-acquisition setup was tested using a stepped neutral density filter, and found to be linear with light intensity for all three colors within 2%, over the whole dynamic range of the camera, (correlation coefficients of the OD with grayscale values for red, green and blue: $R>0.996$; $R^2>0.993$). The images were captured with 20X (lung tissue) and 40X (breast tissue) objective lenses.

Image processing

The 24-bit RGB images were transferred to a Macintosh G4 (Apple Computer, Cupertino, California, USA) and processed and analyzed using NIH image version 1.62, developed at the National Institutes of Health (NIH) and available on the internet from http://rsb.info.nih.gov/nih-image/. Custom macros were written for background correction and transformation from intensity to optical density (OD), to determine the color-vectors for the different stains, for calculation of the color-deconvolution matrix, and for the actual color-deconvolution of the images. The stored image of an empty field was used for determination of the light entering at each pixel ($I_{o,c}$), implicitly correcting for unequal illumination background subtraction.

Test of the system performance

To test the performance of the deconvolution algorithm, different staining combinations were quantified on the same areas of tissue sample. Quantification of DAB staining was

performed by repeated measurement of samples stained with only DAB, DAB plus hematoxylin, and DAB plus hematoxylin plus eosin at different levels. To test the influence of eosin staining on the quantification of hematoxylin, repeated measurements were performed with several levels of eosin staining. The interaction of the stains with respect to quantification of each separated stain was compared using the analysis of covariance. This was accomplished using SPSS (SPSS, Inc, Chicago, USA) and the custom program AOC (www.odin.mdacc.tmc.edu). This procedure follows the general plan described by Zar[12]. Descriptive comparison of performance was done using the measures mean-shift, scale-factor (the ratio of the standard deviations), precision (equal to the correlation coefficient), accuracy (a mixture of the means and the standard deviations), and concordance (product of precision and accuracy) as described by Fisher and van Belle[13]. With optimal reproducibility, the mean-shift centers around 0, the scale-factor centers around 1, and the precision , accuracy, and concordance maximize to 1.
.

4. Results

Figure (1) show a representative example of DAB, hematoxylin and eosin staining before and after color-deconvolution. As can be seen, the result is a set of three images showing continuous representation of the three contributing stains.
To test the performance of the color-deconvolution algorithm under different circumstances, we compared quantification of the stains using different combinations and concentrations. Descriptive comparison of performance was done using the measures mean-shift, scale-factor (the ratio of the standard deviations), precision (equal to the correlation coefficient), accuracy (a mixture of the means and the standard deviations), and concordance (product of precision and accuracy) as described by Fisher and van Belle[13]. With optimal reproducibility, the mean-shift centers around 0, the scale-factor centers around 1, and the precision , accuracy, and concordance maximize to 1.
Addition of hematoxylin staining to the slides did not result in significant changes in the measured IOD of the DAB staining (figure 2). The mean-shift of the IOD measurements was less than 0.05, and the scale-factor was more than 0.95. With a precision of more than 0.99, this resulted in an accuracy of more than 0.99 and a concordance of more than 0.99.
When hematoxylin and eosin both were added to the DAB stained slides, no difference in slope was observed, ($p=0.281$) but a significant mean-shift ($p<0.001$) was seen (figure 2). This indicates a difference in level, but no interaction of stains that would change the rate of quantification of DAB. Although after addition of eosin a significant mean-shift to higher IOD values was observed, the scale-factor was 0.95. Because of the high precision (0.99) and the high accuracy (0.98) the resulting concordance (0.97) still was high.
To further study the influence of the eosin staining on the DAB quantification, we also measured the DAB IOD with different levels of eosin counterstain. Eosin staining was added, or 'bleached' by incubating the slides in 95% alcohol or distilled water, until appreciable reduction in the eosin staining was observed. This way, eosin concentration was increased and reduced to about 75% and 40% of the initial concentration. There were no significant differences in slope ($p=0.738$) or mean ($p=0.193$) of the DAB quantifications, indicating that the three eosin levels did not significantly interfere with the DAB quantification (figure 3). Even after these multiple rounds of processing, the DAB measurements showed precision, accuracy and concordance of more than 0.99, with mean-shifts of less than 0.1, and scale-factors of 0.98-1.00. These data clearly show the stability of the stain and the robustness of the quantification procedure.
Finally, to study the interaction between hematoxylin and eosin quantification, we stained and destained sections of lung tissue stained with hematoxylin, and different levels of eosin. The results of these experiments are shown in figure 4. Quantification of hematoxylin decreased after manipulation of eosin staining. The first eosin staining mainly resulted in a mean-shift of the measurements to lower values, with a mean-shift value of 0.59, while the scale-factor was 1.06. With a precision of more than 0.99, this resulted in an accuracy and concordance of 0.84.
To determine whether this was the result of interference of the colors in the quantification process, or came from actual reduction of the staining due to technical artifacts (bleaching of the hematoxylin during eosin staining), we repeatedly destained and

restained the samples with eosin. The slopes were significantly different ($p=0.0096$) with the change showing a dose/response relationship with the repeated treatments (adding or bleaching) of eosin, reducing the slope (0.943 to 0.792, resp) versus hematoxylin alone. This happened regardless of whether the eosin staining increased or decreased. Therefore, we conclude that the decrease in hematoxylin measurements is the result of reduction of the amount of stain during processing, due to bleaching in aqueous media, not due to interference of quantification of the two stains.

5. Discussion

Image analysis for the quantification of differential cytochemical or immuno-histochemical staining has the advantage over biochemical assays and flow cytometry that it is non-destructive, and that information concerning the relation between morphology and the cell organelle or protein under study is maintained. In addition, automated analysis is faster, and more objective and less laborious than visual examination. Segmentation and density measurements can be performed using interactive as well as computer-determined thresholds. However, overlap in spectral absorption by the stains used can be a major problem.

Several methods have been proposed in attempts to get independent information about the stain concentrations in histological and cytological specimens. Some of the systems either try to limit the spectral overlap of the stains (monochromatic dyes) or the spectral overlap of the detection system (narrow band filters). The use of monochromatic dyes is limited by availability of such dyes. The use of narrow band filter requires sometimes expensive filters, and results in reduced signal, which may compromise the signal-to-noise ratio of the image, and thereby reduce the reliability of the measurements. Even with the use of narrow-band filters overlap in absorption in the specific wavelength regions may occur, resulting in incomplete elimination of the respective chromogens[7]

Another category of methods uses the basic RGB information, or a transformation of this information into e.g. HSI or color-specific components, for segmentation of the image in areas of different colors. These color separation techniques use one of the transformed vales (e.g. hue or chroma) for color classification by some thresholding technique. The classification of pixels is exclusive; each pixel is designated as one of the targeted colors or hues. However, as absorbtion of different colors contributes to the overall red, green and blue absorption, and as such also to the overall hue, saturation and intensity, color separation can no longer be reliably achieved.

We present a color deconvolution algorithm, which uses the information of all contributing colors. It can be used on standard RGB images, as acquired with a standard three-channel analog or digital camera. Processing can be performed quickly and easily on a Macintosh computer using custom macros written for the public domain program NIH image. This means that for the price of a good 3-chip CCD camera and a digitizer board, it is possible to perform quantitative histochemical analysis.

The present method of color deconvolution allows the separate presentation of stain components, even if the stains show overlapping spectral absorption spectra as well as co-localization. Measurement of the color-vectors of the specific stains can easily be determined on single stained specimens. In case of interaction between stains, the user can select areas of the multiple-stained slides as representative of the color to be measured. The method overcomes the disadvantages of filtering techniques or RGB or HSI color segmentation techniques. The color deconvolution technique allows insight into the biochemical composition of a tissue, because it leads to the accurate quantification of the relative distribution of each stain.

As shown above, addition of hematoxylin or eosin to the DAB staining hardly influences DAB quantification. Eosin staining also doesn't seem to influence the hematoxylin quantification, apart from the histotechnical complication of bleaching of the stain.

Because hematoxylin and eosin are soluble in aqueous media, it was not possible to reliably determine the influence of addition of DAB to eosin or hematoxylin quantification. However, we did apply the above algorithm to DAB only stained sections, to make an estimate of the possible (erroneous) contribution of DAB staining to the hematoxylin and eosin quantification. Erroneous deconvolution may especially be the result of saturation of staining and signal detection. As it is clear that saturation of a signal makes it impossible to do accurate quantification, all color separation techniques including the present one, will result in erroneous measurements when satuartion takes place. Therefore, extremely dark DAB staining may result in erroneous measurement of 'hematoxylin' signal and 'eosin' signal, even if these color are not actually present. We had extremely dark DAB staining in some areas of the slides; an example is given in figure 6. As can be clearly seen, the very dark areas of the DAB stained slide also contribute to the hematoxylin and eosin image. As a result of this, possible contribution of DAB saturated areas to the measured IOD for hematoxylin reached an average of 65%. Eosin quantification was less influenced by this phenomenon; only about 8% of the IOD measured at the lowest eosin staining level could possibly be attributed to saturation of the DAB staining, while the mean amount was only 3% for the darkest eosin staining. The present data show that chromogen quantification is not significantly influenced by addition of a second or third color, but mainly by the stability of the chromogens used in the staining procedure, as long as saturation of staining in each of the colors is prevented. This means that using the presented color separation technique, the reliability of the measurements depends mainly on the quality of the sample input, stressing the importance of the use of standard methods, inclusion of standard samples, and prevention of signal saturation.

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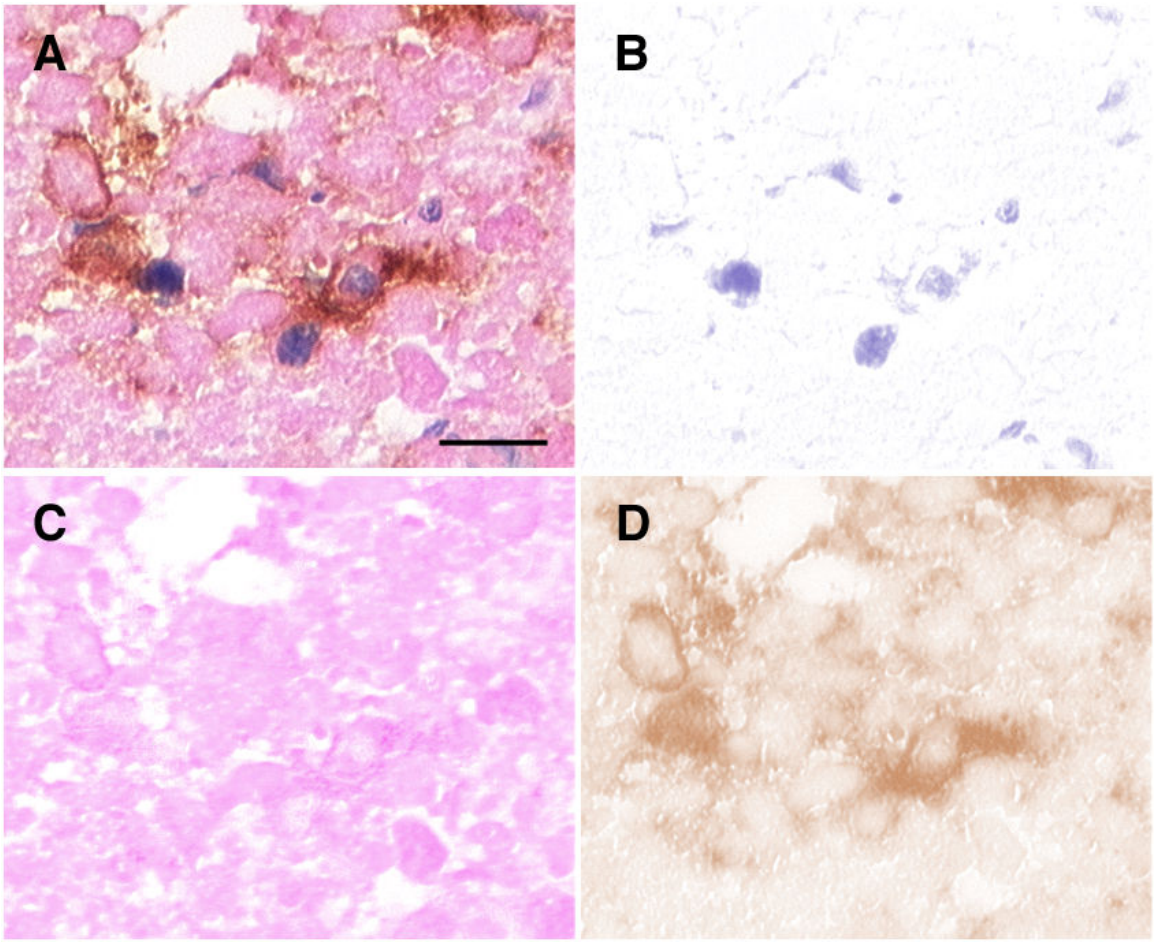

Figure 1: (A) Photomicrograph of a breast tissue slide that is stained with DAB (brown), hematoxylin (blue) and eosin (magenta). (B-D) Color-deconvolution results separating the contributions of hematoxylin (B), eosin (C) and DAB (D) to the original image. Magnification: 40X; bar:20µm.

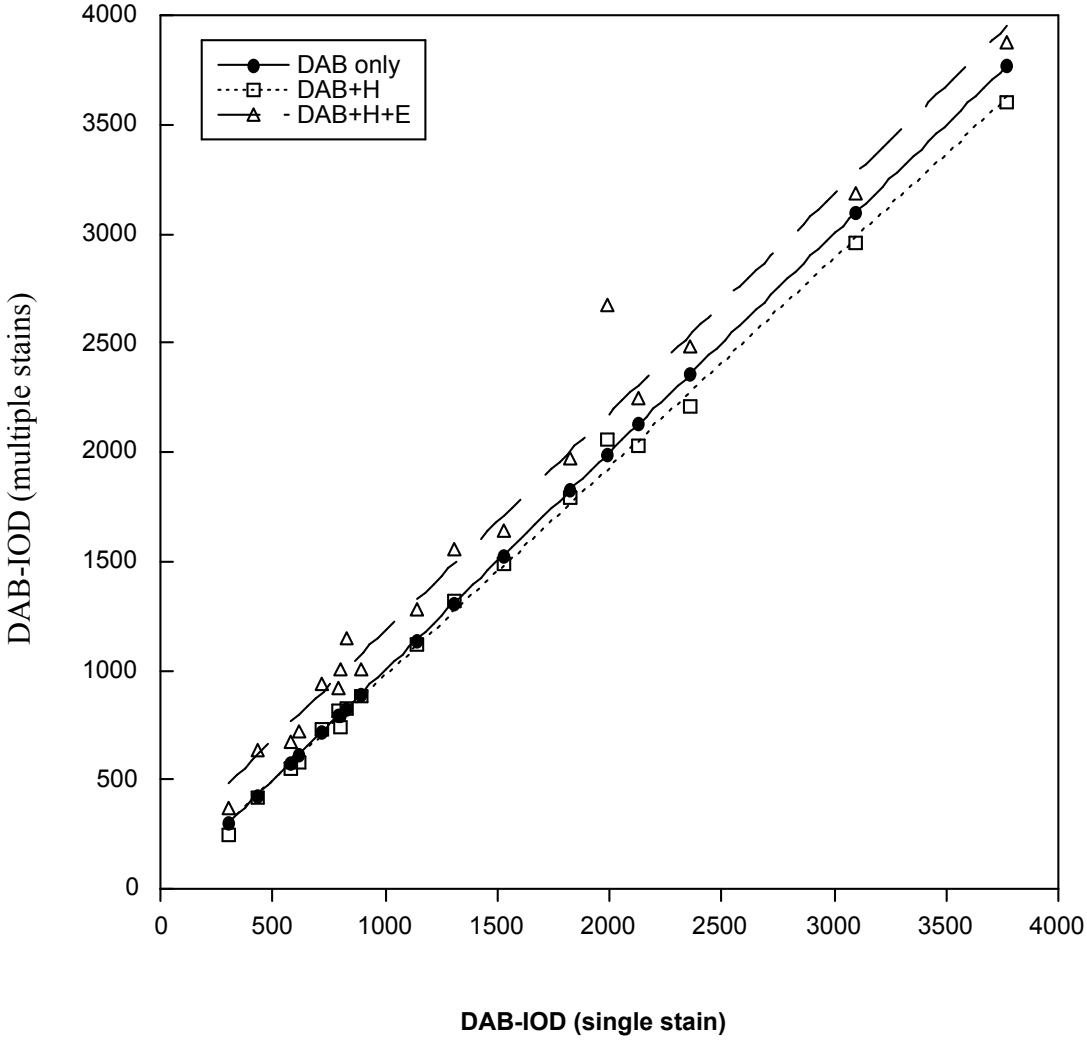

Figure 2: The influence of combination of hematoxylin and eosin on the quantification of DAB in breast tissue sections. The IOD measurement for DAB in areas stained with different chromogen combinations was plotted against the IOD measurements for DAB staining alone in the same areas.

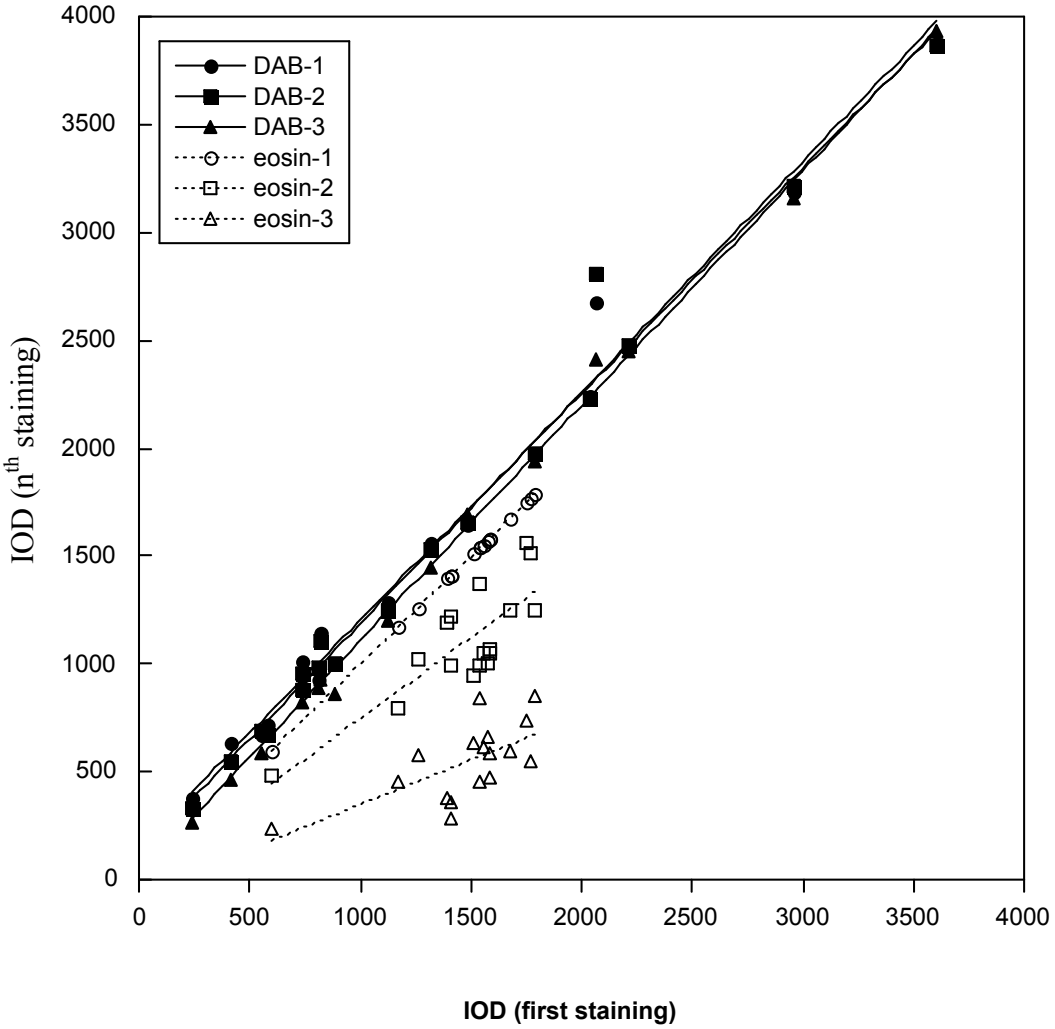

Figure 3: The influence of different levels of eosin staining on the quantification of DAB and eosin in breast tissue sections. The IOD measurement for DAB and eosin in areas stained with different amounts of eosin was plotted against the IOD measurements for DAB and eosin staining (first staining) alone in the same areas. In the first eosin staining step eosin was added (100%), in the following two steps increasing amounts of eosin were 'bleached' from the slide (to about 75% and 40%).

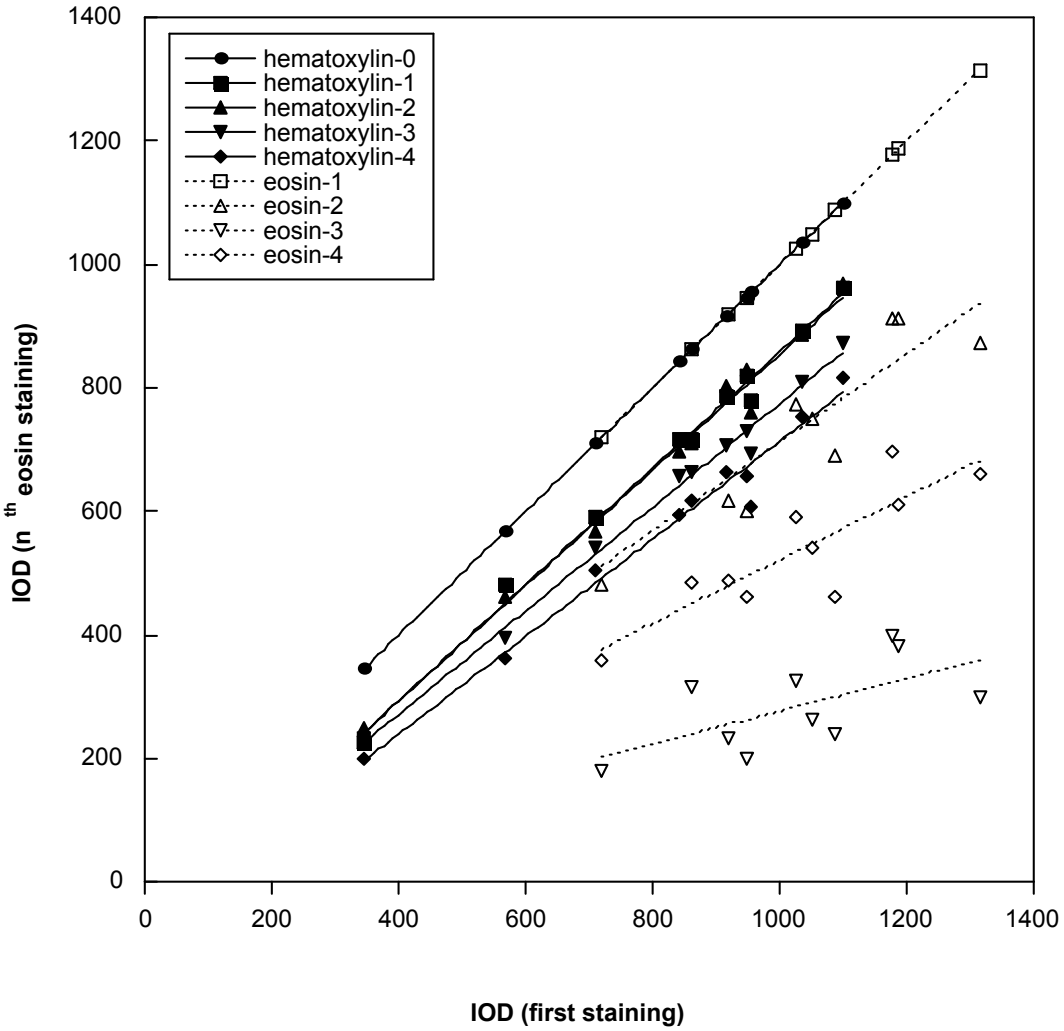

Figure 4: The influence of different levels of eosin staining on the quantification of hematoxylin in lung tissue sections. The IOD measurement for hematoxylin and eosin in areas stained with different amounts of eosin was plotted against the IOD measurements for DAB and eosin staining (first staining) alone in the same areas. In the first eosin staining step eosin was added (100%), in the second and third steps increasing amounts of eosin were 'bleached' from the slide (to about 71 and 27%), in the fourth step eosin was added again (to about 51%).

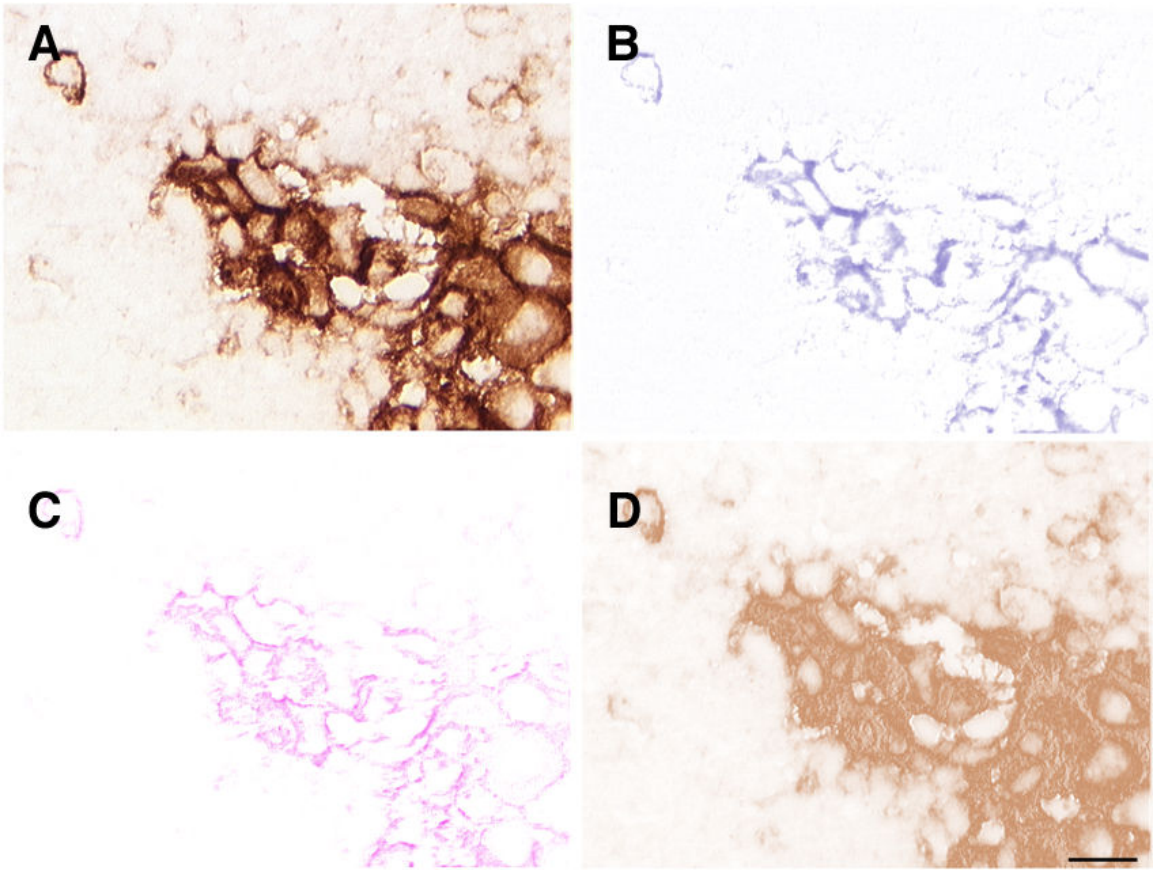

Figure 5: (A) Photomicrograph of a breast tissue slide that is stained with saturating levels of DAB (brown). (B-D) color-deconvolution results, showing the apparent contribution of hematoxylin (B) and eosin (C). Magnification: 40X; bar:20µm.