Color Correction of Red Blood Cell Area in H&E Stained Images by Using Multispectral Imaging

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Abstract

The color of stained pathological images varies depending on staining conditions. Since pathologists based their diagnosis on changes in color and morphology of a particular tissue component, it is important that color variations in stained pathological images be corrected so as to obtain a more reliable diagnosis. A color correction for Hematoxylin & Eosin (H&E) stained images was proposed previously. However, the method did not successfully correct the color of red blood cell (RBC) but only the nucleus and cytoplasm. In this paper, a modified color correction method that can appropriately correct the color of RBC areas in H&E stained images is proposed. In the method, a different weighting coefficient was assigned to RBC areas, which was based from the averaged dye amount ratio in RBC areas in reference and test slides, and a new basis function was derived, in addition to the original three bases functions, to represent the change in the absorption coefficient in RBC areas. In the experiment, the effectiveness of the proposed method was shown with respect to the color difference between reference and test slides in CIE-LAB space.

Introduction

In pathological diagnosis, pathologists examine the microscopic patterns in tissue slides to determine how cell and tissue morphology has changed, so as to yield the final diagnosis and the subsequent treatment policy. Tissue sections are stained to enhance its contrast because original tissue sections are almost colorless; H&E stain is commonly used for routine diagnostic procedures. Since most staining reactions involve a chemical union between dye and stained substance, the color of stained tissue sections could be determined by staining conditions such as the staining time, the temperature or pH of the solution[1,2]. However it is difficult to keep the staining conditions constant in practice. Moreover, different staining-condition standards are often used depending on facilities or pathologist. In addition, if the pathological image is captured by a digital camera, the color of the image is also affected by the characteristics of the microscope and the imaging device. As a result, the color of pathological images acquired by different time or facilities largely varies in the present circumstance. Such color variation becomes a problem especially in virtual slide[3-5,] and telepathology systems[6-8], in which pathological images acquired by various facilities should be examined. Therefore, in order to perform reliable diagnosis, a color correction method is strongly required.

Most slides including H&E stained slides are stained by multiple dyes and they are prepared through multiple staining procedures. In the color correction for pathological images, it is required to perform the color correction corresponding to each staining procedure independently.

Recently, multispectral microscopic systems have been implemented to acquire the spectral transmittance and developed for the image analysis for diagnosis support [9-18]. There are a number of spectral imaging technologies known as acousto-optic tunable filters [9], liquid crystal tunable filters [10], Fourier transformed interferometry [11], and variable interference filter [18]. Moreover it has been reported that spectral information obtained from multispectral images makes it possible to estimate the amount of dyes bound to tissue in the multiple stained slides [17,18].

By utilizing the multispectral imaging technology, a color correction method for pathological images in which the estimated amount of dyes are directly corrected and the corresponding color images are generated has been proposed[20-23]. This method enables the color correction of a test image with respect to a target image of optimal staining-condition.

However in the previously proposed color correction method, the corrected color in RBC areas is not close enough to the color of RBC areas in the target image as compared with cell nuclei and cytoplasm areas. This color difference in RBC areas is considered to be due to a change in the spectral characteristics in red blood cell which is related to the chemical reaction between the cell and eosin stain[21]. This can also be due to the weighting coefficient assigned in red blood areas which should have been different from cell nuclei and cytoplasm areas.

The color of RBC is related to the state of hemoglobin and changes in its color may signal a disease. Therefore, in this paper, a modified color correction method that can appropriately correct the color of RBC areas in H&E stained images is proposed. In the method, a different weighting coefficient was assigned to RBC areas, and a new basis function was derived to represent the change in the absorption coefficient in RBC areas.

The proposed method is applied to H&E stained liver slide, and the colorimetric accuracy of the corrected images is evaluated from pathological diagnosis viewpoints provided that a multispectral image captured by a microscopic multispectral camera with a 16-band rotating filter wheel is utilized.

Original Color Correction method

We briefly review the color correction method of H&E stained image by using multispectral imaging proposed in [20-23].

Image acquisition

The multispectral microscope developed by Telecommunication Advancement Organization, Akasaka Natural Vision Japan was used to capture the tissue sample subjects. The microscope system is composed of a rotational filter with 16 interference filters whose spectral response spans the visible spectrum region; 2000x2000 pixels CCD camera; an OLYMPUS BX-62 optical microscope controlled by a PC with a video grabbing board [16]. The possible extension of the filters' operating region to the near infrared region (NIR) is a subject reserved for future investigation.

Transmittance data

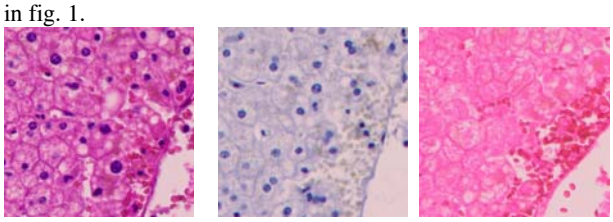
The multispectral microscopic imaging system employed in the experiment comes with software that enables interactive selections of the above mentioned histologic components,i.e nucleus, cytoplasm, and RBCs; the estimate of their transmittance spectra at location **r** is calculated as follows:

$$T(\mathbf{r},\lambda) = \frac{I_o(\mathbf{r},\lambda) - I_{do}(\mathbf{r},\lambda)}{I_{ref}(\mathbf{r},\lambda) - I_{dref}(\mathbf{r},\lambda)} \quad (1)$$

Where $T(\mathbf{r}, \lambda)$ refers to the transmittance spectra of the selected component at location **r**; $I_O(\mathbf{r}, \lambda)$ is the intensity signal of the object image; $I_{ref}(\mathbf{r}, \lambda)$ is the reference signal; $I_{dref}(\mathbf{r}, \lambda)$ and $I_{do}(\mathbf{r}, \lambda)$ refer respectively to the dark current signals of the reference image and the object image. The reference image is acquired by imaging a slide with no tissue sample on it, while the dark current image is obtained with no illumination of the CCD camera.

Dye amount correction

Unstained tissue slides are almost completely transparent and only red blood cells possess coloring pigments. Therefore, after H&E staining procedure, there are mainly three kinds of pigments in the slides; H, E and that of red blood cell. In H&E staining, H stains the nuclear region blue to purple and E stains the cytoplasm, connective tissues etc. pink to red. The H&E stained image, H stained image and E stained image are shown in fig. 1.



(a) (b) (c)

Figure 1. *color image of slide stained by :(a)H&E : (b)H only (c): E only*

To reveal the quantitative relation between the spectral transmittance and the amount of dye in a stained slide, the Beer Lambert law is applied[17-19]. If Beer Lambert law can be applied, we have

$$-\log\{T(\mathbf{r},\lambda)\} = \sum_{i=H,E,R} \varepsilon_i(\lambda) C_i(\mathbf{r}) \quad (2)$$

where $t(\lambda)$ is the spectral transmittance; $C_i(\mathbf{r})$ represents the amount of dye at location **r** and $\varepsilon_i(\lambda)$ represents the spectral absorption coefficient of the *ith* dye along the optical path. The amount of dye $C_i(\mathbf{r})$ is estimated through least square method. Correction of the dye amounts is carried out by introducing a weighting factor to the above equation, and thus we obtained the corrected spectral transmittance as follows:

$$-Log\{T'(\mathbf{r},\lambda)\} = \sum_{i=H,E,R} w_i \varepsilon_i(\lambda) C_i(\mathbf{r}), \quad (3)$$

where w_i is the weighting factor for the *ith* dye. The expression that governs the calculation of the weighting factor is:

$$w_i = \frac{\langle C_i(\mathbf{r}_{tar}) \rangle}{\langle C_i(\mathbf{r}_{test}) \rangle} \quad , \quad (i = H,E,R) \quad (4)$$

where $C_i(\mathbf{r}_{tar})$ and $C_i(\mathbf{r}_{test})$ refer respectively to the amount of dye in target image and test image;<> represents the average over the selected area. The w_i is estimated from the ratio of the average dye amount in cell nuclei areas and stay constant for all location. By using this weighting factor, only the color in cell nuclei and cell cytoplasm areas was properly corrected.

Color reproduction from spectral transmittance

To visualize the resulting color corrected image, the 16-band transmittance spectrum is converted to its equivalent RGB values [23]. A colorimetric image is obtained by color imaging equation as

$$x'_j(\mathbf{r}) = \int M_j(\lambda) E(\lambda) T'(\mathbf{r},\lambda) d\lambda \,, \quad (5)$$

where $x'_j(\mathbf{r})$ is one of the tristimulus values corresponding to color matching function $M_j(\lambda)$, such as CIE 1931 XYZ. The image for display can be generated from the tristimulus image by taking into account the characteristics of the display device.

Modified Color Correction Method

In this section, the modified color correction method that can correct appropriately the color in RBC areas is explained. The ratio of H and E dye amount in nuclei and RBC areas is different and unlike nuclei areas the dye absorption change in RBC areas cannot be represented by three bases as calculated using Beer-Lambert Law. Thus, in this paper, we proposed a color correction method for the RBC areas by considering the following modifications of the original color correction method:

Modification 1

Firstly, for RBC areas we used the ratio between the averaged dye amounts in RBC areas in target slide and test slide as the weighting coefficient w_i^{RBC} (eq.4),and for areas that do not belong to RBC we used the ratio between dye amounts in cell nuclei areas as the weighting coefficient w_i^{nuc}.

$$w_i = \begin{cases} w_i^{nuc} & without\ RBC\ area \\ w_i^{RBC} & RBC\ area \end{cases} \quad , \quad (i = H,E,R) \quad (6)$$

Modification 2

To correct appropriately the color in RBC areas, we make a modification to eq. (3): a new basis function $\varepsilon_{R^*}(\lambda)$ that represents the change in the absorption coefficient in RBC areas is introduced to the equation:

$$-Log\{T'(\mathbf{r},\lambda)\} = \sum_{i=H,E,R} w_i^{RBC} \varepsilon_i(\lambda) C_i(\mathbf{r}) + w_{R^*}^{RBC} \varepsilon_{R^*}(\lambda) C_{R^*}(\mathbf{r}) \quad (7)$$

where w_{R*}^{RBC} and $C_{R*}(\mathbf{r})$ represent a weighting coefficient and a amount of the new basis function.

In this paper, the original color correction method along with modifications 1 and 2 form our proposed color correction method. With these modifications the color in RBC areas can be corrected appropriately without influencing the color of other tissue components. The new color correction process is illustrated in fig. 2.

Experiment and results

H&E Tissue slides

In this experiment, four slides of H&E stained liver specimens were prepared under different staining conditions: normal, over, excess H, and excess E. Over stained slides were prepared with longer staining time respectively, for both H and E stains. The slide of excess H is intensively over-stained with H, while stained normally with E. The specimens on the slides belong to the continuous sections of one tissue and have the same thickness. The H&E stained slide labeled as normal is defined as target slide with optimal staining condition.

The absorption coefficient

To obtain the absorption coefficients of H, E and red blood cell, $\varepsilon_i(\lambda)$, we prepared three slides, a slide stained by H only, stained by E only and unstained, and the optical densities of these slides were measured by using microscopic spectrophotometer (MCPD-3000). Since it is difficult to obtain the absolute spectral absorption coefficients, the normalized optical densities, shown in Fig.3, were used as spectral absorption coefficient instead.

The new basis function

The new basis function that represents the change in the absorption coefficient in RBC areas was obtained from the spectral residual component resulting from the estimation of the amount of dye by means of least square error. The first principal component of the spectral residual component serves as the new basis function. Figure 4 shows the new basis function.

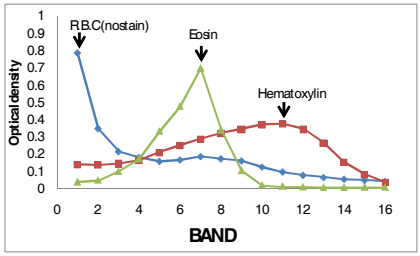


Figure 3. *Normalized spectral absorption coefficients of hematoxylin, eosin and red blood cell*

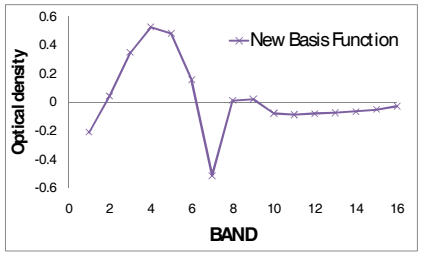


Figure 4. *The new basis function that represents the change in the absorption coefficient in RBC areas*

The effect of new basis function

To examine whether the spectral transmittance can be validly expressed by Eq.(3) and Eq.(7), we define the two kind of normalized mean square error (NMSE):

$$NMSE(H,E,R)=\frac{\left\langle \int_{vis.}\left(T(\lambda,\mathbf{r})-Exp\{-\sum^{H,E,R}\varepsilon_i(\lambda)C_i(\mathbf{r})\}\right)^2 d\lambda\right\rangle}{\left\langle \int_{vis.}t(\lambda,\mathbf{r})^2 d\lambda\right\rangle} \quad (8)$$

$$NMSE(H,E,R,R*)=\frac{\left\langle \int_{vis.}\left(T(\lambda,\mathbf{r})-Exp\{-\sum^{H,E,R,R*}\varepsilon_i(\lambda)C_i(\mathbf{r})\}\right)^2 d\lambda\right\rangle}{\left\langle \int_{vis.}t(\lambda,\mathbf{r})^2 d\lambda\right\rangle} \quad (9)$$

Where $T(\mathbf{r}, \lambda)$ is estimated the transmittance spectra by eq.(1). Table 1 and 2 shows respectively NMSE(H,E,R) and NMSE(H,E,R,R*) of the nuclear area, the cytoplasm area, and the red blood cell area for all H&E slide images. For each tissue component, 200 points were used to calculate the both NMSE. In table 1, NMSE(H,E,R) is especially high for the red blood cell area of excess H and excess E, and thus it is difficult to compose the spectral absorbance of the dyes using eq. (2). On the other

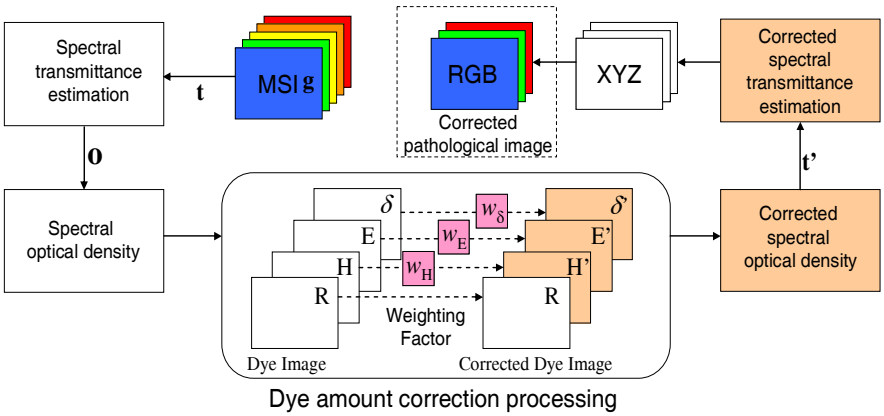


Figure 2. *The process of the modified color correction method in RBC areas based on multispectral imaging technique and Beer-Lambert law.*

hand, NMSE(H,E,R,R*) become small for all tissue component. This result indicates that the spectral transmittance in RBC areas is represented by introducing new basis function.

Table 1: NMSE(H,E,R) for all H&E slide images

NMSE (H,E,R)	Optimal	Over	Excess H	Excess E
Cytoplasm	0.14%	0.24%	0.29%	0.20%
Cell Nuclei	0.45%	0.85%	1.20%	1.03%
RBC	1.35%	0.69%	**1.72**%	**4.79**%

Table 2: NMSE(H,E,R,R*) for all H&E slide images

NMSE (H,E,R,R*)	Optimal	Over	Excess H	Excess E
Cytoplasm	0.13%	0.23%	0.27%	0.14%
Cell Nuclei	0.50%	0.63%	0.72%	0.38%
RBC	0.68%	0.39%	0.39%	0.91%

The estimated dye images

Figure 5 shows the estimated amount of each dye for H&E slide labeled as excess E on a 600×600-pixel area; Fig.5(a) is the color image generated from the 16-band multispectral image and Figs.5(b)-5(d) are the three decomposition images corresponding to the amount of H, E and red blood cell respectively; the grey-level of each pixel corresponds to the amount of each dye: black to zero and white to the maximum. In Fig.5(a), purple circle regions correspond to nucleus, small darker pink regions correspond to red blood cells, and remaining lighter pink regions corresponds to cytoplasm. We can see that the H stains mainly the nucleus area in Fig. 5(b); the E stains the nucleus, the cytoplasm and red blood cell regions in Fig.5(c); white regions correspond to RBC areas in Fig. 5(d). These results agree well with pathological assessment.

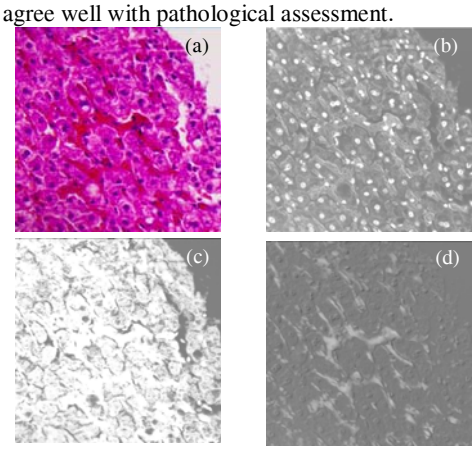


Figure 5. *Images generated from the multispectral image of a liver tissue: (a) color image of the tissue; the multispectral image is decomposed into images corresponding to the amount of: (b) hematoxylin (c) eosin (d) red blood cell*

The separation between RBC areas and other tissue component

The RBC image explained above is converted into a binary image in Fig.(6) by using a threshold to separate between the RBC areas and the other tissue component. The threshold is experimentally determined. In Fig.(6), white region and black region respectively represent RBC areas and the other tissue components. In the new method, the original correction method is applied to the black region and the proposed modifications; i.e. modifications 1 and 2, is applied to the white region.

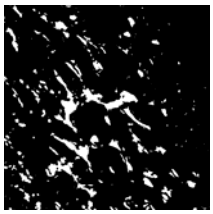


Figure 6. *binary image of amount of red blood cell.*

Color Correction

A normal stained tissue slide was used as target slide, and the averaged color difference between target slide and corrected test slide in CIE-LAB color space was calculated. Table 3 shows the averaged color difference in RBC areas in uncorrected images, corrected images by original method and proposed method, respectively. It can be observed that the proposed method consistently results to smaller color difference for all cases which is in contrast to the results of the original method. Figure 7 shows the color corrected H&E image for excess E stained tissue slide. With the original color correction method, the color of RBC areas becomes darker than the reference. However with the proposed method, the color of RBC areas is closer to the reference.

These results show the effectiveness of the proposed method over the original method in correcting the color of RBC areas.

Table 3: Averaged color difference of RBC areas in CIE-LAB

RBC Areas	Over	Excess H	Excess E
Uncorrected	16.99	8.17	9.96
Original method	11.72	10.69	11.93
Proposed method	1.10	3.09	3.72

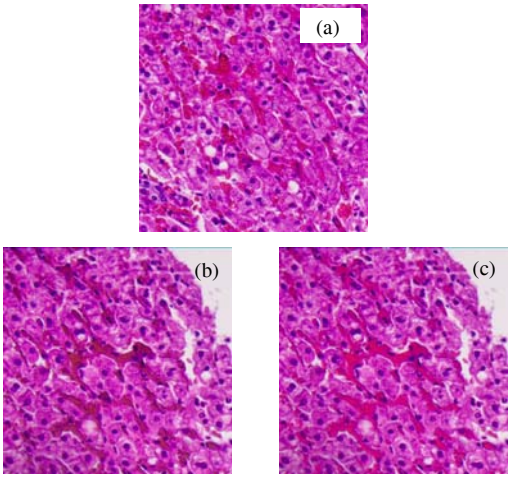


Figure 7. *(a)Color image of normal H&E stained tissue as target image, color corrected image of Excess E stained slide:(b)by original method; (c)proposed method*

Conclusions

We have proposed a new color correction method that can appropriately correct the color of RBC areas in

H&E stained images. To realize this, the method utilizes a different weighting coefficient for RBC areas and new basis function that represents the change in the absorption coefficient in RBC areas. Through our experiments using an H&E stained liver slide, we confirmed that the proposed method can appropriately correct not only the color of cell nuclei and cytoplasm areas but also RBC areas. Although we used 16 band images in this method, we could further reduce the number of multispectral images if accurate statistical information of spectral transmittance were obtained in advance. In future work, we will study this using a number of slides and also adapt this method for other organs.

Acknowledgment

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