An Image Pre-Processing Improvement Procedure for Standardizing Multiple Colour Variations in the Astrocytoma Immunohistochemical Staining Images

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Abstract. Colour variations in the histopathological images can occur when the acquired images came from different laboratories. This issue happens may be due to different protocols between each laboratory. Besides that, the usage of dye or staining is also different since it arrived from different manufacturers. Hence, it will affect the performance of a system, especially for an automated image diagnosis system. If this issue is taken lightly, the whole process of image analysis can produce a false diagnosis outcome, and thus, it will affect the patient's treatment options and endanger the patient's life. This paper proposed an automated image pre-processing procedure for standardizing the colour variations in immunohistochemical staining images. The proposed procedure was done by modifying the reference image, which will be used later for the stain standardization process. Based on 50 astrocytoma histopathological images, the proposed procedure showed a promising result, where the quality of the output images has been enhanced and standardized.

1. Introduction

Image pre-processing is an essential technique in digital image analysis. This technique is fundamental and one of the preliminary tasks necessary for achieving high accuracy and relevant results. The image pre-processing technique improves the original image data by removing undesired distortions and enhancing the targeted features for better visualization. In histopathological images' applications, the image pre-processing technique is inevitable due to several issues arising in the original images, such as colour and intensity inhomogeneity, non-uniform illumination conditions, colour variations from specimen slides, and the presence of noise and artefacts. Thus, it is vital to perform this technique since it will influence the outcome of the analysis.

A brain tumour refers to a collection of abnormal neoplastic cells within the brain, which can be benign or malignant. Neoplasia can be defined as abnormal growth and proliferation of abnormal tissues that may develop into a tumour [1]. The benign tumour usually grows slowly, and these abnormal cells often remained at the original site [2]. For malignant tumours, these cells can invade new tissues or metastasize to other distant organs [2]. In 2020, the American Cancer Society estimated 23890 new malignant brain and nerve tumours diagnosed in the United States [3]. Astrocytoma is a type of primary brain tumour that arises in the star-shaped cell (astrocytes) that acts as supportive tissues in the brain.

Staining is a procedure that involves staining specimens with specific dyes to visualize cell structures and components under a microscope. The variation of colour staining is a common issue in histopathological image analysis. This issue usually will occur when the digitized image came from different scanners, different staining protocols, and different manufacturers who produced the stain
colouring or dyes [4]. This issue is very challenging, especially for an automated image analysis system, because this colour variation may lead to the misclassification of cancerous cells. Ki67 immunohistochemical (IHC) staining is a procedure used for detecting an antigen in a sectioned tissue by using antibodies. This staining is widely used to determine tumour grading and an effective method in assessing the prognosis in several tumour types. For this staining type, diaminobenzidine (DAB) will be used to stain the sample tissue for visualizing the immunopositive Ki67 or tumour cells and counterstained with Haematoxylin (H). As a result, the immunopositive Ki67 cells will appear in granular brown, whereas the immunonegative Ki67 cells or normal cells will appear blue. From these cells’ appearance, it is proved that colour is the significant feature for differentiating between cancerous and non-cancerous cells. Therefore, it is a need to reduce the colour variability issue in order to ensure good accuracy and efficacy of the diagnostic system.

Stain normalization, sometimes called stain standardization, is a pre-processing technique that aims to standardize the histological stains in digital pathology. Recent studies have shown several techniques can be used to standardize the staining variability. The Reinhard algorithm is one of the well-known and suitable techniques for resolving the aforementioned issue. However, the shortcoming of this technique is that the output image is truly dependant on the target image. The contrast of the output image will be degraded if the contrast of the source image is higher than the contrast of the target image [5]. Consequently, it will affect the performance of the diagnostic system. Thus, this study proposed an improved algorithm based on the Reinhard algorithm to solve the target image issue and hence the resultant image contrast issue. The following section will discuss the previous studies related to the application of image pre-processing techniques. Section 3 will explain in detail the procedures and methodology to develop the proposed automated system. Section 4 presents the analysis results, and Section 5 concludes the paper.

2. Stain Normalization and Standardization Techniques

Previous studies have shown the importance of stain standardization in histopathological image analysis. It is necessary and significant to perform this technique in the early stage of the diagnosis system as it will affect the system’s performance.

Eycke et al. [6] had adapted two previously proposed procedures for standardizing colour based on IHC staining. These techniques involve of Macenko algorithm and the Complete Colour Normalization algorithm. In their study, a slice from a reference tissue microarray (TMA) was added to each IHC batch to serve as a staining reference. The next step is to obtain the optical density values from each RGB intensity channel. The authors improved the conversion procedure by measuring the intensity of the glass slide background for illumination normalization. The following step is to extract the colour vector from the TMA image and using these information values in the colour deconvolution process. An additional step has been added to the Macenko algorithm to improve the fitting of the optical density value distributions between staining batches. The normalization staining results were compared to each other for performance analysis. As a result, the Macenko algorithm outperformed the Complete Colour Normalization algorithm. The authors concluded that the application of the Complete Colour Normalization algorithm increased noises, which makes the staining detection became less robust.

Sainz de Cea and Nie [7] proposed a framework that can normalize an arbitrary single stain from a stained histologic specimen. For this study, the authors are focusing on normalizing the haematoxylin stain from the IHC staining. Initially, the system will identify and separate the pixels into two classes, which are pure haematoxylin pixels and haematoxylin stain mixture pixels. Each category is processed by using different approaches. For pure haematoxylin pixels, the system will convert these pixels from RGB to HSD (Hue-Saturation-Density) space. Then, the authors used the chromatic and density information from a selected template image to align with the colour and density distribution of the haematoxylin stain in the targeted image. Finally, the system performed the inverse HSD transformation and converted it back into an RGB image. The next process is the haematoxylin stain mixture pixels. At first, the system applied the unmixing technique to the template and target images. This process is to separate between the DAB and haematoxylin channel. Then, the system will utilize the mean and standard deviation information from these two images to align the color and density distribution. Subsequently, the transformed haematoxylin pixels are recombined with the DAB pixels to form an RGB image. The final result showed that the proposed method can provide consistency in haematoxylin stain through different images without affecting the DAB stain.
Roy, Lal. And Kini [5] introduced a colour normalization algorithm for Haematoxylin and Eosin (H&E) Stained images. The proposed algorithm is based on the conventional Reinhard algorithm, where this algorithm has been modified by incorporating the fuzzy logic for normalizing the stained images. Normalizing the stained colour by using the conventional Reinhard algorithm was done in the L* a* b* colour space. Therefore, the modification algorithm was done by utilizing the fuzzy logic to control the contrast enhancement in L* space and control the colour co-efficient in a* b* space to reduce colour variation. The proposed algorithm was tested on two different datasets, which consist of breast and colon cancer. The proposed method showed significant results for both datasets, where the mean Pearson Correlation Coefficient (PCC) obtained were 0.9998 (breast cancer) and 0.9988 (colon cancer).

Lakshmanan, Anand, and Jenitha [4] discussed the performances of three existing colour normalization techniques (Reinhard algorithm, Macenko algorithm, and Histogram Specification) to be applied on breast cancer H&E stained images. The performance of these techniques was evaluated by calculating the Structural Similarity Index Metric (SSIM) and Entropy quality metrics. According to the SSIM analysis, the Reinhard algorithm produced the highest value compared to other techniques with 0.93. The entropy’s value for the normalized image using the Reinhard algorithm also outperformed the other two techniques. The authors also presenting the comparison of computational complexity results in this study. Based on the analysis for 150 stained images, the Reinhard algorithm was the fastest and had the lowest execution time, with an average of 59.8 seconds.

3. Automated Image Pre-Processing Procedure

This paper focuses on developing an image pre-processing procedure for standardizing multiple colour variations in IHC staining images. Figure 1 presents the flowchart of proposed algorithm.

3.1. Image Acquisition

There were 50 astrocytomas IHC staining images with various colour appearances used in this study. These images were captured at the Department of Pathology, Hospital Universiti Sains Malaysia (HUSM). These images were captured under 40x magnification using an Olympus BX51 microscope and Cell^F software that acts as an interface to the digital camera attached to the
microscope. The size of each captured image was 4140×3096 pixels. The captured images were then saved in (*.jpg) format and 24-bit RGB.

3.2. Modified Staining Normalization Algorithm
Typically, stain standardization (normalization) is the foremost approach to solve the stain colour variances problem. Stain standardization is a technique of changing the range of pixel intensity values by transforming the mean colour of an image to another image. Generally, a target image is needed when performing this technique. The selection of a target image is crucial since it can affect the whole process of the analysis. Besides, when having a large of image datasets, this process might take longer to make decisions. In this study, the target image was selected based on two criteria: (i) good contrast and staining intensity that can distinguish between each cell and (ii) good visibility of the cells’ textures. Since the target image is necessary for this technique, the modification will focus on modifying the target image into an “ideal” stained image.

At first, the proposed system will calculate the contrast for both source and target images. As mentioned in Section 1, the quality of the output image will be degraded if the contrast of the source image is greater than the contrast of the target image. The contrast’s values were obtained by applying a no-reference image quality algorithm, which has been proposed by Yan, Li, and Fu [8]. This algorithm is a metric that assesses the quality of the image’s contrast based on five features. Initially, the proposed algorithm will enhance the input image by using the histogram equalization technique. Then, the structural-similarity index (SSIM) between the original and enhanced images was calculated to obtain the first feature. Next, the histogram-based entropy and cross-entropy were measured for original and enhanced images respectively, to gain a total of four features. The selected features will be learned through a Support Vector Regression (SVR) module to compute the quality score. A higher score indicates the image has better contrast quality.

The next step is to compare the two contrast values. In this situation, two conditions need to be highlighted. For the first condition, if the contrast of the target image is higher than the contrast of the source image, the proposed algorithm will make a little adjustment to the target image by increasing the brightness of the target image to acquire the optimum results. Therefore, the colour space of the target image will be converted from RGB to HSI colour space first. Then, the Logarithmic Transformation is applied to the intensity (I) channel to enhance the image’s brightness. Next, the modified target image was converted back into RGB colour space. An unsharp masking technique will be applied to sharpen the image.

The second condition arises when the contrast of the target image is lower than the contrast of the source image. For this situation, the proposed algorithm will apply a histogram matching technique to match the histogram between the source and target images. Similar to the first condition, the modification process will be applied at HSI colour space. Thus, the colour space of the source and target images will be converted into HSI colour space first. The HSI colour space model may differentiate between intensity and chromaticity. The chromaticity can be expressed as hue (H) and saturation (S). The hue reflects the dominant colour as perceived by a human, while saturation refers to the purity of colour. As the contrast of the output image will be decreased in this second condition, the proposed algorithm will restore the contrast by applying the histogram matching technique at the $S$ channel. Hence, a new saturation image will be created by transforming the histogram of the source image so that its histogram will match with the histogram of the target image. The brightness of the image is then increased by using the Logarithmic Transformation on the $I$ channel. Subsequently, each of the channels is combined and converted back into RGB colour space. For better visualization, an unsharp masking technique will be implemented to improve the quality of the image.

3.3. Stain Standardization
For this study, stain standardization was done by applying a method call Reinhard Stain Normalization. This standardization process works by using the mean and variance from both reference and target images and transferring the colour from the reference image to a source image. The selection is made based on the advantage of this technique that preserved the source image's structure. Another advantage of this technique was that the processed image's contrast would be similar to the reference image. The process was done as follows:
Convert the colour space for both (source and modified reference) images from RGB to $L^*a^*b^*$ colour space.

Carry out the following transformation in $L^*a^*b^*$ colour space:

\[
L_2 = \mu_g(L) + \left[ L - \mu_g(L) \right] \frac{\sigma_g(L)}{\sigma_g(L)}
\]

\[
a_2 = \mu_g(a) + \left[ a - \mu_g(a) \right] \frac{\sigma_g(a)}{\sigma_g(a)}
\]

\[
b_2 = \mu_g(b) + \left[ b - \mu_g(b) \right] \frac{\sigma_g(b)}{\sigma_g(b)}
\]

where $L_2$, $a_2$, and $b_2$ are intensity variables of the processed image in $L^*a^*b^*$ colour space. $L_1$, $a_1$, and $b_1$ refer to the intensity variables of the reference image in $L^*a^*b^*$ colour space. $L$, $a$, and $b$ indicate the intensity variables of the source image. $\mu_g$ is the global mean of the image, while $\sigma_g$ is the global standard deviation of the image.

Convert back the colour space of the processed image into RGB colour space.

3.4. Quantitative Measurement

In this study, eight quantitative measurements were used to measure the performance and the output image's quality. These measurements consist of Tenengrad Criterion (TEN), Feature Similarity Index (FSIM), RMS Contrast, Universal Image Quality Index (UIQI), Structural Similarity Index (SSIM), Virtual Information Fidelity (VIF), Absolute Mean Brightness Error (AMBE), and Correlation Efficient. The details of the measurement as follows:

- **Tenengrad Criterion** is a measurement to measure the sharpness of an image. A higher value of TEN suggests sharper edges [10].

- **Feature Similarity Index** values inform about the feature similarity between two images, which is based on low-level features. Higher FSIM value implies better performance [11].

- **RMS Contrast** is used to measure the contrast of an image. Higher values of RMS Contrast indicate the image has better contrast [12].

- **Universal Image Quality Index** is a measurement that quantifies image distortion between the original image and the modified image. The measurement is consists of three components: loss of correlation, luminance distortion and contrast distortion. This model varies from -1 to 1. Higher value demonstrate greater similarity between the two images [13].

- **Structural Similarity Index** quantifies the structural loss based on statistical moments. The range of index values is between -1 to 1. A value of 1 indicates both images are identical [14].

- **Virtual Information Fidelity** is used to calculating the loss of image information. The default value is 1. If the VIF value is less than 1, it signifies a loss of visual quality in the processed image (degraded). If a VIF value that more than 1, it indicates the quality of the image has been improved [15].

- **Absolute Mean Brightness Error** is a brightness preservation assessment that quantifies the difference in the mean brightness of the input and modified images. A good brightness preserving method will have a low AMBE value [16].

- **Correlation Coefficient** is a method that measures the degree of linear relationship between the original and processed images. Both original and processed images are considered absolutely identical, if the correlation value, $r = 1$ [5].

4. Result and Discussion

A personal computer that runs on an Intel Core i7-5500U, 2.4 GHz, a processor with 16.0 GB RAM was used to develop the system. The proposed algorithm was built by using MATLAB version 2018. 50 astrocytoma IHC staining images were tested in this study. Figure 2 illustrates several resultant images produced using the proposed algorithm and the other two global standardization techniques: the conventional Reinhard algorithm and Histogram Specification.
Figure 2. Comparison results of processed images between the application of original and modified reference images to the image pre-processing procedure.

Figure 1 had demonstrated several source images with varying illumination and staining intensity. Generally, the proposed algorithm provided better illumination output images compared to the other global techniques. The target image in Figure 1 was the original image with no adjustments or modifications applied to it. In this study, the proposed algorithm enhanced the brightness of the target image, which results in better quality and visualization of the output image. This can be observed at the immunopositive Ki67 cells’ morphology, where the visibility of the cell’s texture and structure has been improved compared to other output images, which have a darker appearance. From the observation in Figure 1, the output image presented using the histogram specification technique does not well-preserved the DAB staining colour from the source image. The third row of resultant images showed only the Reinhard algorithm and the proposed algorithm preserve the source DAB staining intensity variation. The fourth source image is one of the examples where the contrast of the image is higher than the contrast of the target image. After applying the stain standardization techniques, the quality of the resultant images performed by the Reinhard algorithm and histogram specification is decreased. This can be seen from the low staining intensity produced at the immunonegative Ki67 cells, which degrades the image's clarity. Compared to the resultant image presented using the proposed algorithm, the contrast of the immunonegative Ki67 cells was better than the other two techniques. This explained that the implementation of the histogram matching technique could enhance the low contrast images. The
following result will present the mean values acquired from eight quantitative measurements for 50 astrocytoma images.

Table 1. Comparison mean values between the source image and the resultant images from two global standardization techniques and the proposed algorithm

| Quantitative Measurement | Source Image | Reinhard Algorithm | Histogram Specification | Proposed Algorithm |
|--------------------------|--------------|--------------------|-------------------------|--------------------|
| TEN                      | 410.4579     | 732.0151           | 1089.9296               | 692.2584           |
| FSIM                      | -            | 0.9686             | 0.9437                  | 0.9718             |
| RMS Contrast             | 0.6844       | 0.6423             | 0.6423                  | 0.7081             |
| UIQI                      | -            | 0.9903             | 0.9902                  | 0.9938             |
| SSIM                      | -            | 0.9689             | 0.9623                  | 0.9728             |
| VIF                       | -            | 1.1719             | 0.9961                  | 1.1419             |
| AMBE                      | -            | 11.3368            | 11.3748                 | 10.0010            |
| Correlation Coefficient   | -            | 0.9932             | 0.9648                  | 0.9951             |

*The best value is highlighted in bold.*

Table 1 presents the mean values of eight image quality assessments performed on 50 astrocytoma histological images. TEN analysis serves as a standard benchmarking to measure the image’s sharpness. A higher TEN value suggested the edges of the image were slightly improved. Table 1 shows the histogram specification acquired the highest TEN value, with an average of 1089.9296. However, as shown in Figure 1, this technique also enhanced the background staining, which increased the noises and may sometimes also bring artifacts in the output images. This occurred since this technique is forcefully stretching the source image's histogram until it approximately matches the target image histogram [17]. The next analysis is focusing on the similarity between the source and output images from each technique. This study employed three different types of assessment to measure the similarity between the two images, which comprise FSIM, UIQI, and SSIM. The FSIM is a feature-based assessment, whereas the calculation of UIQI was related to the image distortion. The SSIM measurement is based on the loss of structural information. The noise level is associated with these three tests. If the noise level is increasing, the quality of the output image will deteriorate. From the results displayed in Table 1, the proposed algorithm achieved the highest result from all three tests. This indicates the output images produced from the proposed algorithm are much similar to the source images with less noise.

For contrast evaluation, the proposed algorithm procures the highest mean value, with an average of 0.7081. Compared with the two techniques, the contrast values are lower than the source image. This happened due to several images which have better quality than the target images, which affects the visual quality results of the output image. VIF is a quality metric that measures the loss of human-perceivable information in an image. The value must be not less than 1 to prevent the quality of the output images is decreased. From Table 1, only Reinhard and the proposed algorithm gave acceptable results with an average of 1.1719 and 1.1419, respectively. The following analysis is AMBE. This quality assessment is designed to evaluate brightness preservation. According to Table 1, the proposed algorithm had the lowest mean value, with an average of 10.0010. This indicates the brightness of the output images produced by using the proposed algorithm is preserved. A decent stain standardization algorithm must preserve all the information of the source image. The correlation coefficient is an appropriate method to measure the information preservation between the source and output images [5]. It is observed in Table 1 the proposed algorithm achieved the highest correlation value compared to other techniques. This value reflects that the two images are highly related and have similar spatial patterns.

5. Conclusion
This paper proposed an automated image pre-processing procedure for standardizing colour variations in IHC images. The proposed procedure consists of several steps, such as contrast calculation, histogram matching, intensity transformations, and applying stain standardization. This study is an improvement from the global stain standardization technique, the Reinhard algorithm. Since this technique relies on the target image, an improvement has been made by modifying the target image to produce better

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results. The implementation of histogram matching has able to recover the low contrast image produced from the conventional technique. The effect of this modification shows a promising result, where the automated procedure is free from the image enhancement technique. Hence, it will reduce the computation time of the analysis. The result also showed the quality and contrast of the source image had been improved. In the future, the number of tested images may be expanded, especially the low-contrast images. It has been discovered that the proposed algorithm has the potential to enhance the cells with low-staining intensity. Next, the proposed procedure may be remodelled to improve the effectiveness, which will help in producing a faster and accurate segmentation technique.

Acknowledgments
The author would like to thank the pathologists from the Department of Pathology, Hospital Universiti Sains Malaysia (HUSM) for helping and contributing to this study.

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