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An introduction to double stain normalization technique for brain tumour histopathological images

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ABSTRACT

Stain normalization is an image pre-processing method extensively used to standardize multiple variances of staining intensity in histopathology image analysis. Staining variations may occur for several reasons, such as unstandardized protocols while preparing the specimens, using dyes from different manufacturers, and varying parameters set while capturing the digital images. In this study, a double stain normalization technique based on immunohistochemical staining is developed to improve the performance of the conventional Reinhard's algorithm. The proposed approach began with preparing a target image by applying the contrast-limited adaptive histogram equalization (CLAHE) technique to the targeted cells. Later, the colour distribution of the input image will be matched to the colour distribution of the target image through the linear transformation process. In this study, the power-law transformation was applied to address the over-enhancement and contrast degradation issues in the conventional method. Five quality metrics comprised of entropy, tenengrad criterion (TEN), mean square error (MSE), structural similarity index (SSIM) and correlation coefficient were used to measure the performance of the proposed system. The experimental results demonstrate that the proposed method outperformed all conventional techniques. The proposed method achieved the highest average values of 5.59, 3854.11 and 94.65 for entropy, TEN, and MSE analyses.

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1. INTRODUCTION

A primary brain tumour refers to a tumour that originates and grows within the brain. Nowadays, the number of patients diagnosed with brain tumours is particularly concerning. According to the American Cancer Society's statistical report, the number of estimated new cases for the brain and other nervous systems increases year after year. In 2022, they estimated there would be 25,050 cases, rising from 2021 to 24,530 cases [1], [2]. The primary brain tumour can be divided into two types, which are glial and non-glial tumours [3]. A glial tumour is a tumour that originates in the glial cells. The glial cells are the glue-like cells responsible for supporting and protecting neurons [4]. A non-glial tumour is a tumour that starts to arise from cells in the brain, which are not categorized as glial cells.

Astrocytoma is a common type of glial tumour, where the tumour arises from star-shaped cells called astrocytes. These astrocyte cells function as supportive tissue within the brain, where it has a variety of functions such as providing nutrients to neurons, supplying the building blocks of neurotransmitters, and

controlling the blood-brain barrier and blood flows [5], [6]. According to the World Health Organization (WHO), astrocytoma can be divided into four grades, which are pilocytic astrocytoma (grade I), diffuse astrocytoma (grade II), anaplastic astrocytoma (grade III), and glioblastoma multiforme (grade IV) [7]. On the other hand, meningioma is one of the examples and most common types of non-glioma tumours. This tumour develops in the three thin layers of tissue surrounding and covering the brain, called meninges [8]. According to the World Health Organization (WHO) grading classification, meningioma is classified into three grades, which are grade I (benign), grade II (atypical), and grade III (malignant) [7].

Cell staining is a technique that utilizes dyes to enhance the cells' morphological visualization and differentiate different tissue structures. With the aid of this staining technique, pathologists and researchers were able to analyses the signs of any disease and predict the treatment outcome that will be delivered to the patient. Colour is central and plays an important role in digital pathology, as it will determine the performance of the automated computer-aided diagnosis (CAD) system. Generally, a new slide specimen prepared by the pathologists will have a good quality colour and intensity. However, the colour staining begins to fade as time goes by, resulting in the cellular components appearing in hazy and feeble colours.

Besides that, in some instances, the staining intensities may appear to differ between each specimen slide. The variance of colour and intensity in the histology slides remains a challenging issue in histopathological image analysis. This issue must be solved earlier before proceeding to the next analysis stages, as the results can affect the accuracy of the CAD system in detecting the targeted cells or tissues. The common reason for this existing issue is probably the inconsistency in the protocols between each laboratory when preparing the slide specimens [9]. In addition, the usage of dyes that arrive from different manufacturers can also lead to this colour variation issue [10]. Yagi [11] stated five major reasons that cause the colour variations, which are the thickness of the specimen, staining, model of scanner used, viewer, and display. Therefore, it is essential to have an adaptive algorithm that can adjust or standardize the preferred colour among the slide specimens to produce a consistent image analysis result.

Stain normalization, sometimes known as colour normalization, is a pre-processing method that aims to reduce the colour and intensity variations by transferring the colour distribution of the source image to the target image [12]. The Reinhard algorithm [13] is a conventional stain normalization approach that has been widely used in previous studies due to its ability to preserve the information in the source image. This algorithm utilizes the linear transformation procedure in the L^*a^*b colour space to enhance the contrast of the source image such that it is approximately equivalent to the reference image. Although the contrast of the cells is substantially improved, it is still inadequate to enhance the cells' contrast in low-quality images. Another standard normalization technique is histogram specification, in which the process is done by mapping the histogram of the reference image to the histogram of the source image. Even though the technique is a straightforward process, it is ineffective if both the reference and source images have significantly different colour distribution statistics. The disparity in both statistics will lead to the existence of image artefacts and excessive noise in the output image. Thus, this paper will introduce a double stain colour normalization method for standardizing the multiple colour variations and improving the visibility of the targeted cells by enhancing the Ki67 cells' features in the histopathological images of astrocytoma. The following section will review in detail the previous works and the conventional methods used in standardizing histopathology images. Section 3 expounds further on the steps to develop the proposed method. Section 4 focuses on experimental results obtained from the proposed method, and section 5 summarizes the paper.

2. PREVIOUS WORKS RELATED TO STAIN NORMALIZATION METHODS

Stain normalization is a pre-processing method that uses the chromatic characteristics of a specific target image to alter the colours of the source image. The implementation of this method reduced the colour and intensity variations, thus improving the automated and quantitative analysis. Based on previous studies, the researchers have developed numerous methods, including the conventional and recent methods to solve staining variations' issues.

Histogram specification is a type of global colour normalization method that follows the global histogram enhancement method for contrast stretching. The normalization process is done by mapping the source image's histogram with the target image's histogram through the histogram equalization enhancement method. As a result, the brightness and colour of the source image will be likely similar to the target image. The advantage of this method is both the brightness and colour statistics of the source image match the target image [9]. Since this method is a histogram-based contrast enhancement method, the histogram of the source image will be forcefully stretched until it is approximately equal to the histogram of the target image. As a result, it may lead to existing artefacts in the processed image while some of the information may be lost and not preserved [10].

Another global method was proposed by Reinhard *et al.* [13], where this method used the mean and standard deviation parameters from the target image and transferred them to the source image. This method was carried out to match the mean and standard deviations of each colour channel between the source and target images by the use of linear transformation in lαβ colour space (that contains the lightness and chromaticity parameters). The main benefit of this method was the structure of the source image was preserved and the contrast of the normalized image was identical to the contrast of the target image [9]. However, the limitation is that the process of transformation in lαβ colour space causes the stains to be improperly separated [10].

Macenko *et al.* [14] proposed a fully automatic algorithm for normalizing histology images based on employing the stain separation method. The authors assumed that each of the two stains in an image had its stain vector and the resulting colour in the optical density (OD) space for every pixel is a linear combination of these two stain vectors. This method was fully automated encompassing few parameters and no optimizations are required. The drawback of this method was the inconsistency issue in producing results when having more than two staining types. Besides that, it is reported that this algorithm does not preserve all information in the source image [10].

Piórkowski [15] presented an improved colour normalization algorithm for improving the accuracy of segmentation and cell nuclei detection in different stained multi-organ tissue. The improvement has been made based on modifying the conventional Reinhard algorithm. The authors manually selected the best potential values from different organ tissues to be implemented in the colour normalization process for better detection. The improvement has been made based on modifying the conventional Reinhard algorithm. The authors manually selected the best potential values from different organ tissues to be implemented in the colour normalization process for better detection. These values are the parameters that have been chosen from each target image, which comprises the mean of red, green, and blue (RGB) channels, the mean from each channel in the lαβ colour space, and the standard deviations of each channel in the *lαβ* colour space. This method produced acceptable results in improving the system for detecting the cell nuclei. However, for images that consist of open chromatin nuclei, the proposed system gave poor detection.

Salvi *et al.* [16] introduced a SCAN algorithm, which was an automated stain separation and normalization algorithm for Haematoxylin and Eosin (H&E) stain histological slides. The SCAN algorithm consisted of four modules, which include preliminary stain separation, cellular structure segmentation, final stain separation, and image normalization. In the preliminary stain separation module, the algorithm performed a white detection by using a series of Gabor kernels for eliminating the uncoloured regions from the input image. Then, Macenko's stain separation method was applied to obtain the initial image of separation between the H&E channels. Next, the algorithm performed a segmentation process to obtain the cellular region of interest, which was the nuclei and stroma. The authors then defined a new colour deconvolution method by using the OD stain values extracted from the segmentation masks to perform the final stain separation. Afterwards, the colour normalization process was performed by using the true stain intensities values from the cellular structures segmentation and the matrix that represented the stain colour appearance from the target image. As a result, the computational time taken by the proposed algorithm was faster compared to other methods. Besides that, the relative square error acquired by the proposed algorithm was also low. In addition, the proposed algorithm was able to increase the percentage of accuracy detection for breast cancer, from 81.59% to 92.87%.

Nowadays, the attention to the application of artificial intelligence (AI) in medical histopathology images is increasing among researchers, and this includes the stain normalization process. Zanjani *et al.* [17] developed a stain normalization model for H&E images by using the generative adversarial network (GAN). The proposed model is comprised of three convolutional neural networks (CNN). These include a generative network, an auxiliary network, and a discriminator network. The generative network functioned to learn how to create a colourized H&E image in the *CIEL*a*b** space by supplying the image lightness channel and a set of structural latent variables that represent colour for image structures. The auxiliary network operated for learning a disentangled latent space by the GANs. The role of the discriminator network was to minimize the loss function defined in the network and attempt to differentiate the generated colour images from the original image. According to the findings of that research, it showed the proposed model outperformed previous stain normalization methods and the normalized image was able to preserve the structures of the source image.

Lou and Le [18] designed a colour correction algorithm to standardize the colour variation in haematology images while preserving the details in the original image. At first, the authors performed a mini-batch k-means clustering to create a colour scheme for both the source and target image. Then, the proposed system generated colour mapping by using B-spline interpolation to map each colour channel for each cluster from the target image to the source image. Next, the system applied the mapping to RGB values of pixels of the source image. In conclusion, the proposed algorithm was able to obtain promising results with the highest structural similarity index (SSIM) results of more than 0.96. The results also showed the mean average

precision (mAP) score used to assess the performance of the system in object detection task were increased by 7% after applying the colour normalization algorithm.

Kang *et al.* [12] introduced StainNet, a distillation learning system that learned colour mapping from the source to target images to perform the stain normalization process. The proposed system consisted of two steps, which are composed of StainGAN training and StainNet generation. The StainGAN worked as the instructor network, where in this step the StainGAN will normalize the images from the source domain to the target domain. The resultant of the normalized images was then used as Ground Truth and will be fed to train the StainNet. StainNet required paired source and target images for the network to learn the colour space transition from the source to the target images. This StainNet is a fully 1×1 convolutional neural network that is applied to extract the mapping relationship from StainGAN. The network used the L1 loss to learn the output from StainGAN. The proposed network produced significant results, where the SSIM result between the normalized and source images was high at 0.945±0.025.

In conclusion, several methods are available for applying stain normalization. The current AI methods show a major advantage over conventional methods, where the usage of the target image for the analysis can be neglected. However, to attain higher performance and high accuracy results with this method, it is essential to have a large amount of dataset so that the network can learn the features directly from various input data. Due to this reason, this paper will propose a stain normalization procedure without using the AI methods since the number of input images is restricted and the limitation issue of the open-source database. The proposed procedure is an improvement and modification from the conventional Reinhard's colour normalization method. The following section will go deeper into how the proposed procedure works.

3. PROPOSED STAIN NORMALISATION PROCEDURE

The idea of developing the proposed stain normalization procedure is based on the conventional Reinhard method. Reinhard's method is a well-known method, especially in normalizing the inconsistency of staining between histopathology images. The selection for using this Reinhard's method as a reference method is based on it is ability to preserve the information of the source image besides enhancing the contrast of the source image so that when the normalization process is applied, the contrast of the output image will be approximately similar to the contrast of the target image. However, selecting the target image is crucial in this method, since it will affect the output image and further processes. Therefore, in this study, an improvement and modification procedure to Reinhard's method has been made to increase the image's quality performance and ease the pathologists' visibility in future analysis.

Previous works in section 2 have shown that most of the proposed methods are applied to good-staining quality datasets. However, it is not reported in the studies about the usage of low-staining quality datasets, which raises concerns about the robustness of the proposed system when using this sort of dataset. These low-staining datasets are useful especially for researchers who are developing the automated diagnosis system. With the availability of these datasets, the amount of the data can be expanded, hence it will improve the robustness of the proposed system in analyzing different image quality. For that reason, this study will focus on developing a stain normalization procedure that is adaptive to various image quality levels. Figure 1 depicts the general block diagram of the proposed stain normalization procedure. The development of the proposed system is based on four primary stages, which are composed of image acquisition, modification of the target image, application of double stain normalization, and enhancement of the cell-targeted features.

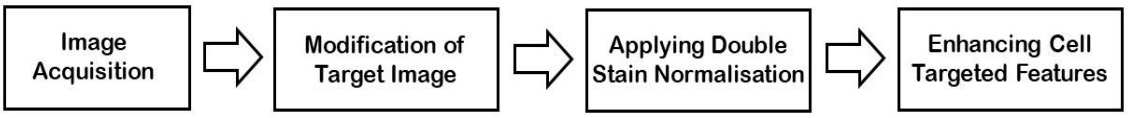


Figure 1. The general flow of developing the proposed stain normalization procedure

3.1. Image acquisition

The datasets utilized in this study were obtained from the Department of Pathology, Hospital Universiti Sains Malaysia (HUSM). The slide specimens were prepared between the years 2013 to 2018. A total number of 60 histopathological images, which comprise two types of brain tumours (astrocytoma and meningioma) were used in this study. These images were captured at 40× magnification using an Olympus BX51 microscope and Cell^F software, which serves as an interface to the microscope's digital camera. The

captured images for astrocytoma cases were 4,140×3,096 pixels in size, while for meningioma cases were 1,360×1,024 pixels. The captured images were then stored in 24-bit RGB (*.jpg) format.

The histopathological images were captured from the Ki67 immunohistochemical (IHC) staining specimens, in which this staining method used antibodies to identify an antigen in a sectioned tissue. Ki67 is a nuclear protein that pathologists frequently used as a prognostic and predictive marker in assessing biopsies from tumour and cancer patients [19]. In Ki67 IHC staining, the sample tissue was stained with Diaminobenzidine (DAB) and counterstained with Haematoxylin. Figure 2 shows a sample of an IHC-stained Ki67 image from an astrocytoma case.

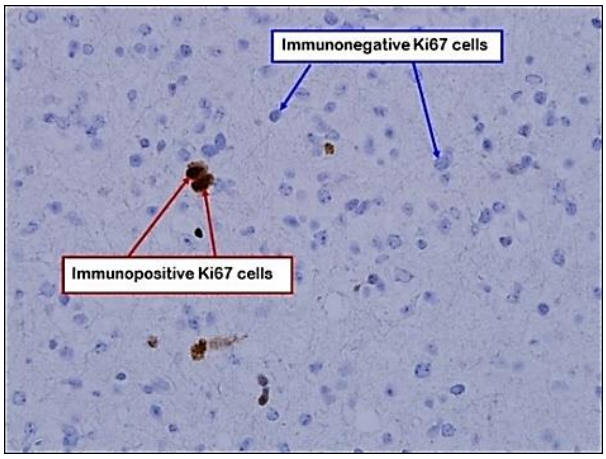


Figure 2. A Ki67 image of astrocytoma using IHC stains

As shown in Figure 2, the result of the IHC staining causes the immunopositive Ki67 cells to appear in granular brown colour while the immunonegative Ki67 cells appear blue. Figure 3 illustrates several histopathological images of brain tumours used in this study with varying image qualities, illumination, and colour staining. These figures demonstrate that the degree of colours varies between each image. The colours in the low-quality images fade, thus reducing the visibility of the morphological features of the cell. Therefore, it is essential to have a pre-processing method to ensure all the input images have a uniform colour for ease of further analysis.

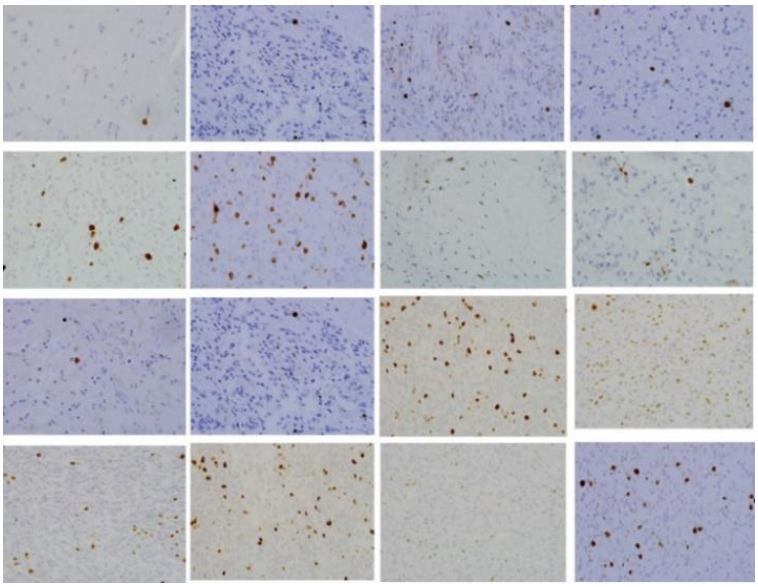


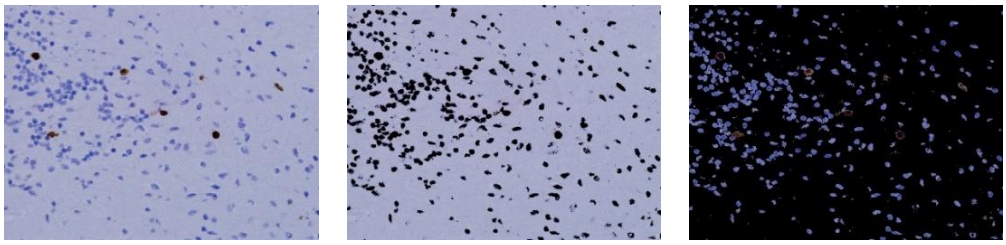
Figure 3. Several histopathology images of astrocytoma and meningioma with different staining intensities and lighting conditions

3.2. Modified target image

In the global Reinhard's method, a target image is required to perform the normalization process. This target image will serve as a reference image, with the target image's colour distribution being used and transferred to the source image. Aside from having an image with good lighting conditions, another criterion that needs to be considered when selecting a target image is the contrast of the target image must be greater than the contrast of the source image [20]. The contrast of the normalized image may be degraded if the quality of the source image is better than the target image.

Conventionally, the selection of the target image is subjective and the selected image is being used without any modification. As a result, the quality of the normalized image may not achieve the best performance because some of the criteria are not met. These criteria may be referring to the contrast of the targeted cells, the visibility of the cell's features, colour temperature, and lighting condition. Cell feature plays an important criterion in determining the tumour grading based on Ki67 expression. Therefore, a modification procedure via the target image will be introduced in this study to enhance the visibility of the cell features in the source image.

Since this study aims to enhance the cell's features visualization, the modification process will focus solely on the cells while maintaining the original background. If the background image is also modified, the system will transfer the entire colour intensity variations from the target image to the source image during the stain normalization process. Consequently, it will lead to the existence of image artefacts [20]. For this reason, the system will separate the target image into three parts, which are the background, immunopositive Ki67 cells, and immunonegative Ki67 cells. At first, the system will apply Otsu's thresholding to separate the background from the foreground objects. After obtaining the foreground objects, the resultant binary mask image was then retrieved back into the RGB colour space for the following process. Figure 4 displays the resultant images after separating the foreground objects from the selected target image. Figure 4(a) depicts the selected target image for modification. Figure 4(b) is the background image that was separated from the foreground objects, while Figure 4(c) is the masked image of the foreground objects.



(a) (b) (c)

Figure 4. Resultant images after separating the foreground objects from the selected target image, (a) selected target image, (b) background's image, and (c) foreground object image

The system will then apply (1) to locate and segment the pixels that represent the redness colour, which is relevant to the colour of the immunopositive Ki67 cell that appears in red-brownish colour. The equation is as (1):

$$f(x,y) = (R) - max\big((G),(B)\big) \quad (1)$$

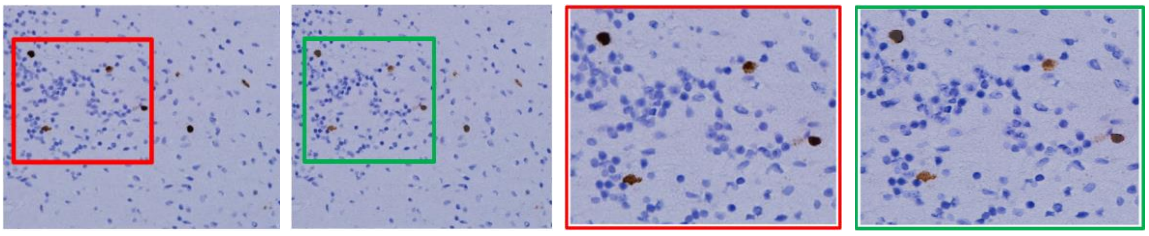
where $f(x,y)$ is the segmented image of immunopositive Ki67 cells. *R*, *G*, and *B* express the pixel values of red, green, and blue channels from the output image acquired from the background removal process. The next process is to convert back the segmented image mask to the RGB colour space by multiplying it with the main RGB image (foreground image from the background removal process). Afterwards is the process of enhancing the contrast of immunopositive Ki67 cells. The contrast enhancement process is usually done by transforming the input image into a colour space that includes image luminosity as one of the components in the colour space. In this study, the RGB image is converted into the *L*a*b** colour space, where this colour space is device-independent and contains all possible colours perceived by the human eye.

As reported in previous studies, there are numerous methods used to enhance the contrast of an image, such as contrast stretching, histogram equalization, adaptive histogram equalization, and contrast-limited adaptive histogram equalization (CLAHE). As for this study, the CLAHE technique was selected since this method can overcome the limitations that existed from the global approaches when performing the

contrast enhancement. When performing the CLAHE technique, two important parameters that require consideration are the number of tiles and clip limit. The number of tiles usually refers to the number of regions or blocks that control the amount of non-overlapping sub-areas [21]. The clip limit is the contrast limit or contrast factor that prevents oversaturation of the image by controlling the noise amplification [21]. The fundamental concept of this CLAHE technique is that the input image is divided into a few tiles first. Then, a standard histogram equalization method is applied to these tiles to enhance the contrast. If the histogram bin is over the specified contrast limit, the pixels will be clipped and distributed uniformly so that the height of the histogram does not exceed the clip limit. Next, the subregions are combined with the bilinear interpolation method to acquire an optimized whole image. The example images in Figure 3 show a difference in contrast and colour intensity between the immunopositive and immunonegative Ki67 cells. Therefore, the CLAHE technique is implemented separately for each type of Ki67 cell. The input image for this study will be the image from the *L* channel, which is separated from the *L*a*b** colour space. The modification at the luminosity channel will affect only the intensity levels at each pixel, while the colours in the image are preserved. The resultant image will be converted back into the RGB colour space.

Subsequently is to enhance the contrast of immunonegative Ki67 cells. The image of immunonegative Ki67 cells can be obtained by subtracting the foreground image (output image after removing the background) from the enhanced image of immunopositive Ki67 cells. The contrast enhancement process is similar to enhancing the contrast of immunopositive Ki67 cells, where the image is converted into the *L*a*b** colour space and applying the CLAHE technique at the luminosity channel. As mentioned in the previous paragraph, the application of CLAHE technique was applied separately to different types of Ki67 cells. The size of tiles is defined similarly for both immunopositive and immunonegative Ki67 cell images, where the images are divided into 8 rows and 8 columns of tiles. However, the clip limit value differs from one another since the contrast and chromatic colour appearance are also varied. For image immunopositive Ki67 cells, the clip limit value was set to 0.2, where this value was the maximum value that preserved all the feature information of the Ki67 cells. A higher value causes more information to be lost, and the image will appear in a dull colour. For image immunonegative Ki67 cells, the clip limit value was simply set to 0.005 since the contrast of the cells in the selected target image was good. The enhanced image is converted back into the RGB colour space. The final modified target image is obtained by combining the background and the enhanced images of immunopositive and immunonegative Ki67 cells. Figure 5 depicts the resultant images of every step involved in modifying the selected target image. Figure 5(a) is the original image, while Figure 5(b) is the new modified image. Figures 5(c) and 5(d) are the zoomed-in images of the original and modified target image.

From the visualization in Figure 5(a), the original target image has good contrast for immunonegative Ki67 cells. However, most of the immunopositive Ki67 cells appear in dark brown, hence confining the system to analyse the cell features within the cell. Compared to the modified target image in Figure 5(b), the contrast of the image is much better since the nucleus for each cell is more visible for both immunopositive and immunonegative Ki67 cells. This enhancement is significant because the modified image's chromatic colour distribution will be transferred to the source image in the following stain normalization step.



(a) (b) (c) (d)

Figure 5. Images of Ki67 cells before and after applying the contrast enhancement technique, (a) original target image, (b) modified target image, (c) the zoomed-in target image before modification, and (d) the zoomed-in target image after modification

3.3. Double stain normalization

The Reinhard standardization method is a method that preserves the intensity variations of the source image by using the mean colour of the target image and transferring it onto the source image. The operation of the Reinhard method is as follows:

- Read the source and target images.
- Resize the source image to a size of 1,360×1,024 pixels. The image resizing is applied to ensure the source image has the same size as the target image.
- Convert the colour space for both images from the RGB into *L*a*b** colour space.
- Calculate the mean (μ) and standard (σ) deviation for every channel in the *L*a*b** colour space for both images.
- Perform the transformation in *L*a*b** colour space:

$$L_2 = \mu_g(L_1) + \left[L - \mu_g(L)\right] * \left[\sigma_g(L_1)/\sigma_g(L)\right] \quad (2)$$

$$a_2 = \mu_g(a_1) + \left[a - \mu_g(a)\right] * \left[\sigma_g(a_1)/\sigma_g(a)\right] \quad (3)$$

$$b_2 = \mu_g(b_1) + \left[b - \mu_g(b)\right] * \left[\sigma_g(b_1)/\sigma_g(b)\right] \quad (4)$$

  where L_2, L_1, and L are the luminosity channels for processed, target and source images respectively. a_2, a_1, and α are the a* channels for processed, target and source images respectively while b_2, b_1, and b are the b* channels for processed, target and source images respectively.
- Combine all the output channels and convert them back into the RGB colour space.

Normally, the process of standardization by using the Reinhard method towards the recent tissue specimens is straightforward. The high contrast produced by the specimens' colour staining has simplified the standardization process. On the contrary, for older specimens with feeble colours and poor contrast visualization, the usage of the conventional Reinhard method is inadequate. Practically, the contrast for these images has been enhanced, but it is still insufficient for analysis. As a result, the first normalization stage is applied, and the resultant image is expected to be comparable to a standard recent staining image without implementing the normalization method yet. Therefore, an additional stage of colour normalization is required to enhance the contrast of the source image. The formulas and steps to perform the normalization are similar to the previous paragraph. The target image used in the second stage of normalization is also alike to the first stage of normalization. The transformation in the second stage works as (5), (6), (7):

$$L_3 = \mu_g(L_1) + \left[L_2 - \mu_g(L_2)\right] * \left[\sigma_g(L_1)/\sigma_g(L_2)\right] \quad (5)$$

$$a_3 = \mu_g(a_1) + \left[a_2 - \mu_g(a_2)\right] * \left[\sigma_g(a_1)/\sigma_g(a_2)\right] \quad (6)$$

$$b_3 = \mu_g(b_1) + \left[b_2 - \mu_g(b_2)\right] * \left[\sigma_g(b_1)/\sigma_g(b_2)\right] \quad (7)$$

where L_3, L_2, and L_1, are the luminosity channels for normalized, target and processed images respectively. a_3, a_2, and a_1 are the a* channels for normalized, target and processed images respectively while b_3, b_2, and b_1, are the b* channels for normalized, target and processed images respectively.

3.4. Enhancing the visibility of cell-targeted features

In the stain standardization process, the contrast of the normalized image should be greater than the contrast of the source image. Nevertheless, the source images used in this study come with multiple variations of colour intensity and different qualities of staining. It is challenging to use the Reinhard method, especially towards images that already have good contrast. Since this study proposes a double stain normalization method, it may have the possibility for the images to have excessive contrast enhancement. Based on the images provided in Figure 3, most of the immunonegative Ki67 cells appear in light blue. The application of double stain normalization to these cells may increase the colour contrast and able to improve the visibility of the features within the cells. Yet, if the contrast of the immunonegative Ki67 cells in the source image is greater than the target image, the quality of the normalized image may be degraded and the contrast of the cells also will reduce. Another situation is for immunopositive Ki67 cells, where most of the cells are present in dark brown, which might be worrisome that the nuclei colour may turn around into darker colour and hence will complicate further analysis. Thus, a power-law transformation method has been implemented in this study to ensure that both of the Ki67 cells will appear in acceptable colour contrast.

The power-law transformation is one of the grey-level transformation types that is commonly used for image enhancement. This transformation maps a narrow range of low grey-level values in the input image into a wider range of output values or vice versa [22]. The transformation is expressed as (8):

$$s = cr^{\gamma} \quad (8)$$

where r and s are the pixels before and after processing respectively. c and γ are positive constants. Parameter c controls the brightness of the input image. As the c value increases, the image's brightness will also increase [23]. In this study, the c value was set to a default value of 1, preserving the brightness level while adjusting the contrast stretching factor, γ. The γ value varies, where different values produce different effects on the image. If γ is less than 1, the output image will appear brighter compared to the input image. γ equals 1 indicates the identity transformation, where the output pixels will have similar pixel values as the pixels before the processing. For γ more than 1, the output image tends to be darker than the previous input image. For this study, the γ should be less than 1 for the immunopositive Ki67 cells image because the objective is to make sure the image of immunopositive Ki67 cells will appear brighter and the nuclei are visible. For the immunonegative Ki67 cells image, the γ should be more than 1 since the aim is to avoid the image degradation issue.

Based on the pre-observation of five images (approximately consisting of 210 immunopositive Ki67 cells and 1,020 immunonegative Ki67 cells), the γ was set to 0.8 and 1.2 for immunopositive and immunonegative Ki67 cells images respectively. These values were the maximum values after considering the naturalness of the image and the appearance of each cell (in terms of contrast and discernible detail for each cell). The contrast enhancement process works as follows: i) The system will segment the double stain normalization image by using (1) to obtain the immunopositive Ki67 cells; ii) Second, the colour space of the immunopositive Ki67 cells was converted from the RGB to *L*a*b** colour space; iii) The power-law transformation was applied at the luminosity channel while preserving the chromaticity channels; and iv) The transformed image was then converted back into RGB colour space. For the immunopositive Ki67 cells image, the process of transformation is similar, where the operation is happening at the luminosity channel of the image. The transformed image is then merged to obtain the final result of the normalization image.

3.5. Quality metrics for histopathological images

Image quality assessment in the pathological field is necessary as it will assist pathologists in analysing and decision-making tasks. The full-reference approach necessitates the use of a reference image to compare it to the test image. However, due to the colour variation that occurred after the staining process, it might be possible that the colour information details will be lost. Hence, the quality metrics used in this study are based on the grayscale information, which was also used by Roy *et al.* [20] as they assumed the grayscale information is entirely preserved in the source image after the staining process.

Five image quality metrics were used in this study to measure the performance and accuracy of the normalized images. First was the entropy analysis, which describes the amount of information contained within an image. A higher entropy value suggests that the image contains more detailed information. The second analysis was to measure the sharpness of an image. Tenengrad criterion (TEN) is a well-known technique based on gradient magnitude maximization [24]. A higher TEN value usually assumes the image as having higher quality and sharper edges. The third analysis was related to the distortion measurement between the source and normalized images. The mean square error-based (MSE) metric is commonly used as an objective measure of distortion by assessing the average squared difference between the pixel values of the source and normalized images [25]. The error refers to the amount by which the values of the source image differ from the processed image. A higher MSE value reflects higher distortion contained within the image. The next analysis focused on measuring the structural similarity between the source and normalized images. For this study, the correlation coefficient and SSIM were used to measure the degree of linear correlation [26], [27]. The correlation value indicates how much the information in the source image is preserved. The higher values of the correlation and SSIM signify higher similarity between the source and normalized images.

4. RESULT AND DISCUSSION

A personal computer with the processor specifications of an Intel Core i7-5500U, 2.4 GHz with 16.0 GB RAM, was used to develop the proposed system. All the programs and algorithms related to this study were executed on MATLAB version 2021b. A total of 60 histopathological images (45 images of meningioma and 15 images of astrocytoma) were tested in this study. Two conventional techniques in stain normalization, which are histogram specification and Reinhard's method, were compared with the proposed method for assessing the performance of each method in standardizing the histopathological images. Figure 6 compares the visual results of the three stain normalization algorithms. The second column until the fourth column represents the normalized images for each method.

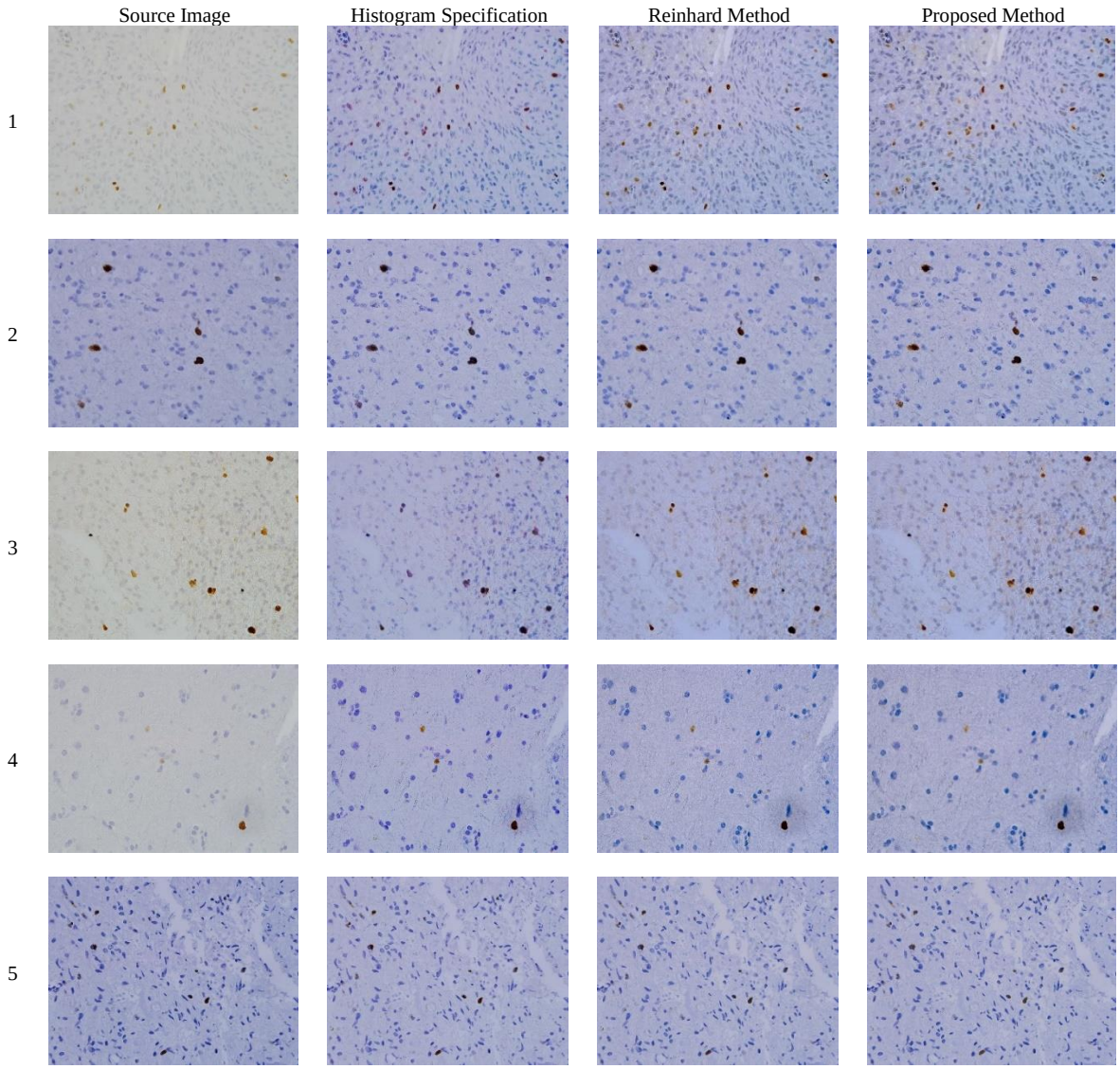


Figure 6. Comparison of various colour normalization methods for meningioma and astrocytoma histopathological images

Based on the results presented in Figure 6, it is shown that all the normalized images approximately have a standardized colour distribution, although the quality of the source images is varied. Besides, the colour intensity for most Ki67 cells has also been increased, albeit some of the cells appear in different colours. For the histogram specification method, it is shown that the contrast of the immunonegative Ki67 cells in the normalized images was mostly better compared to other methods. Despite having the varying quality of source images, the normalized images for this method show the colour of the immunonegative Ki67 cells has been standardized. However, the results produced by this method failed to preserve the information on the immunopositive Ki67 cells in the source image. The normalized images produced by the single stain normalization using Reinhard's method were acceptable. Nonetheless, if the quality of the source image is higher than the target image, the quality and the contrast of the cells produced in the normalized image may be degraded as shown in Figure 6 (row number 5). It is shown that the contrast of the immunonegative Ki67 cells has decreased when using Reinhard's method. Figure 7 shows in detail a comparison of the visualization of the cell features between the proposed and the conventional methods. Figure 7(a) is the source image, while Figures 7(b) until 7(d) are the resultant images from three different methods, which are the proposed procedure, Reinhard's method, and histogram specification.

Based on the depiction in Figure 7(d), the immunopositive Ki67 cells have been transforming into a false-colour image, where the colour of the cells had transformed into a magenta colour. These results could

lead to misinterpretation by pathologists as an image artefact. In addition, as shown in Figure 7(c), there is an excess of contrast enhancement in immunopositive Ki67 cells, which makes the cells appear darker than the original cells in the source image. As a result, some detailed information related to those cells was lost. This contradicts the resultant images produced using the proposed method, where the immunopositive Ki67 cells appear brighter, and the cells' features are more discernable than the Reinhard method. The application of the power law transformation has successfully transformed the appearance of the immunopositive Ki67 cells to become more visible. Besides, the image of the immunonegative Ki67 cells showed a more appealing appearance than the Reinhard method because it has a higher contrast. The following analysis represents the results related to the image quality measurements:

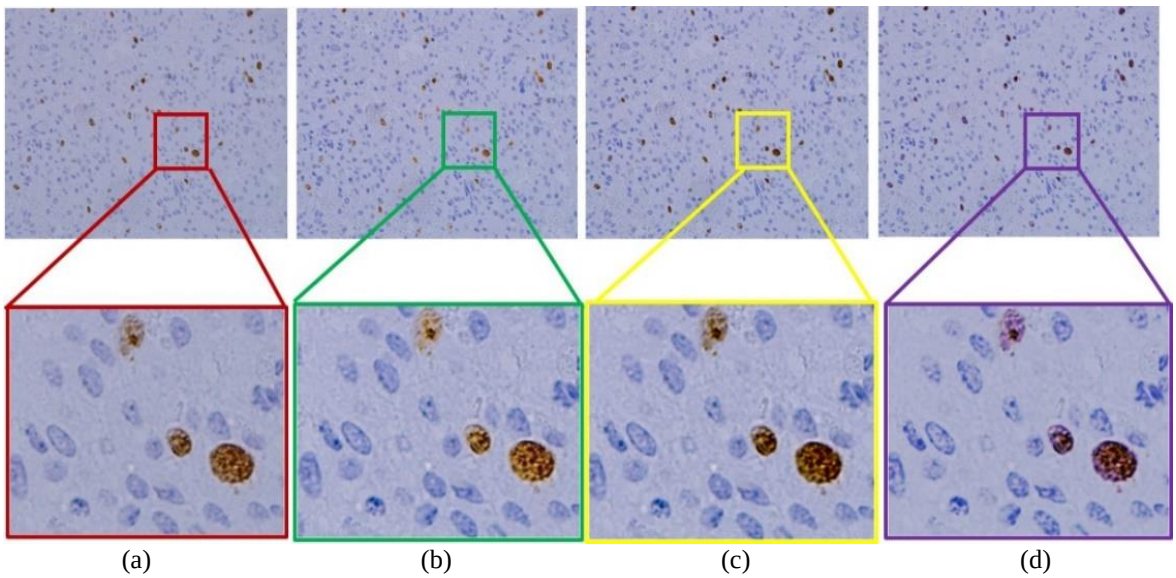


Figure 7. Visual comparison of cell features produced by the stain normalization methods, (a) source image, (b) proposed method, (c) Reinhard's method, and (d) histogram specification

Table 1 demonstrates the average values acquired from five image quality metrics tested on 60 histopathology images. A high entropy value indicates the image is rich in detail. From the results in Table 1, it is shown the proposed method has the highest entropy value of 5.59. The significant contribution to the increase in entropy value came mostly from the image of immunopositive Ki67 cells, whose appearance has been improved to the more visible cells. From the aspect of image sharpness, the proposed method also yields the highest value compared to other methods, with 3854.11. For the MSE parameter, a higher value expresses a higher error. This indicates the processed image has a greater difference when compared to the original image. From the presented results in Table 1, the proposed method was able to obtain an average error value of 94.65. Reinhard's method was able to achieve a high degree of correlation with an average of 0.979. Nevertheless, the proposed method was also able to achieve a promising result of correlation with an average of 0.965. The next quality metric was to measure the structural similarity between the normalized image and the source image. The results in Table 1 reveal the histogram specification method was able to achieve a high SSIM value with an average of 0.908. However, it was obvious from the findings shown in Figure 7 that the colour of the majority of the immunopositive Ki67 cells had been transformed to magenta, which contrasted with the colour of the original cells in the source image. Hence, it can be inferred that this SSIM measure should not be used to assess the structural similarity between normalised and source pictures.

Table 1. Comparison of average values acquired from five image quality metrics

Quantitative measurements	Source image	Histogram specification	Reinhard's method	Proposed method
Entropy	4.84	5.26	5.54	**5.59**
SSIM	-	**0.908**	0.905	0.877
TEN	1427.68	3164.48	3111.20	**3854.11**
MSE	-	95.25	95.89	**94.65**
Correlation coefficient	-	0.948	**0.979**	0.965

5. CONCLUSION

In this paper, a double stain normalization method was proposed for standardizing and enhancing brain tumour histopathology images. This method is an improved version of the global Reinhard stain normalization method. The idea to develop a double stain normalization procedure came from the fact that some of the immunonegative Ki67 cells still had low contrast issues after the global method was implemented into the cells' image. Therefore, the first improvement was focusing on preparing a target image. This study proposed a method to improve the quality of the target image by solely enhancing the contrast of the cells while maintaining the colour intensity of the image's background. This approach is more reliable than the global normalization method that randomly selects a target image to be used for the normalization process. The global target selection image's limitation was time-consuming, and it may increase the interobserver variability among the pathologists since numerous input data images were captured from the tumour specimens. Besides, not all the cells in the target image were high in contrast. Therefore, this study proposed a pre-processing method by modifying the selected target image into a better-quality image before applying the double stain normalization method. The implementation of the CLAHE technique towards the targeted cells has improved the cells' contrast while preserving the background luminance of the target image.

The second improvement was concentrating on the visualization of the cell features. The results presented in the previous section show that the proposed double stain normalization produced significant results compared to the histogram specification and Reinhard's method. The colour intensity produced by the proposed method was higher compared to others, proving that the study's goal was achieved. The proposed method was able to enhance the colour intensity of the Ki67 cells without regard for the quality of the source images. Furthermore, the proposed method was also able to avoid quality degradation and over-enhancement issues that exist in Reinhard's method. The findings showed that the application of power-law Transformation had effectively enhanced the visibility of the Ki67 cell features. As for the immunopositive Ki67 cells, most of the cells that appeared darker after the double stain normalization method have converted into a brighter appearance. Besides, the power law transformation technique was also successful in improving the quality of the immunonegative Ki67 cells' image by darkening some of the cells' images that remained low contrast after implementing the double stain normalization.

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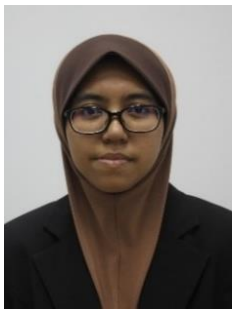

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