Combining Thresholding and Clustering Techniques for Mycobacterium tuberculosis Segmentation in Tissue Sections

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Abstract: This paper presents an automated method for segmentation of Mycobacterium tuberculosis in tissue sections by combining thresholding and clustering techniques. First, a global thresholding is used to eliminate pixels associated with the methylene blue counterstain. Then, k-mean clustering algorithm is applied to remove the ZN-stained pixels which are not associated with the bacilli. The effectiveness of these techniques is evaluated using three colour models; the RGB, the HSI, and the CY. Automatic seeded region growing technique is further applied to eliminate unwanted regions and noises. Finally, the segmented regions are refined by applying a local adaptive thresholding and smoothed using a median filter. 100 images of tissue slides were tested and verified by comparing with the manual-segmentation image. By using the saturation of CY colour model, the framework produced the best segmentation performance, achieving accuracy, sensitivity, specificity and Jaccard coefficient value of up to 99.41%, 81.44%, 99.71% and 0.641, respectively.

Key words: Image segmentation, thresholding, clustering, tuberculosis bacilli detection, tissue sections.

INTRODUCTION

Tuberculosis, commonly known as TB, is a communicable disease caused by Mycobacterium tuberculosis infection. Although curable with antibiotics treatment, TB remains the second killer disease after HIV/AIDS. It is estimated that 9.4 million new cases of TB and 1.68 deaths due to TB worldwide, and mostly in the low and middle-income countries (WHO, 2010). TB usually affects the human lung causing pulmonary TB (PTB), yet there are also cases where it strikes other parts of the human body, referred as extra-pulmonary TB (EPTB).

TB is diagnosed by detecting the presence of Mycobacterium tuberculosis in patient samples. Currently, a number of established methods are used for the TB diagnosis such as microscopy, chest X-ray, tubercul skin test (TST), interferon-γ release assay (IGRA), serology and nucleic acid amplification technique (NAAT) (Tiwari et al., 2007). However microscopic examination using light microscope is a common and widely used due to low technical cost, rapid, and easy to perform. For PTB, the diagnosis is conducted by the sputum examination, while for EPTB, the biopsied tissue of the infected organ is used for diagnosis. Clinical specimens are stained using the Ziehl-Neelsen (ZN) method to visualize TB bacilli in the sample.

Early detection and initiation of effective treatment are important in reducing the incidence and mortality from TB disease. The results of smear microscopy are recommended to be reported within 24 hours while TB bacilli detection and classification within 10-14 days (CDC, 1995). In clinical practice the rapid detection of individuals with TB can be difficult. The manual screening of TB is time-consuming and tedious, especially for detecting negative slides. Furthermore, an accurate diagnosis requires an assessment conducted by well-trained personnel. However, in low and medium-income countries with high TB incidence, the large number of slides must be interpreted and diagnosed by a relatively small number of medical staff. These problems pose a huge obstacle in obtaining a rapid and accurate result hence efforts to improve the quality of existing methods are necessary.

The rapid progress in computer hardware, software, image processing algorithm and artificial intelligence has led to research and development of computer-aided TB diagnosis system. The aim of the system is to assist medical technologist in the diagnostic process. A number of techniques for automated PTB detection using sputum smear have been proposed in the literature. Some of these techniques used the fluorescence microscopic image (Forero et al., 2004; Veropoulos et al., 1998; Veropoulos et al., 1999) whilst others used light microscopic image (Khutlang et al., 2009; Nayak et al., 2010; Sadaphal et al., 2008).

While most of the researches concerned with the PTB, little research has been done on EPTB detection. The EPTB contributes 15% to 20% of all TB cases and accounts for more than 50 % of the cases in HIV-positive patients (Sharma & Mohan, 2004). Sadaphal et al. (2008) were the first to propose an automatic method for identifying the TB bacilli in both sputum smear and tissue sections. However, only the result on the sputum-
A smear was shown in the report. Tadrous (Tadrous, 2010) proposed an image ranking algorithm to assist medical technologist in searching for TB bacilli. The method used ‘colour score’ and ‘shape score’ to calculate the probability of containing of TB bacilli in an image. Then, the algorithm ranked all the images according to the highest probability of the presence of bacilli. However, the method needs medical technologist to determine manually the positions and regions of TB bacilli in a tissue slide image. More recent work by Osman et al. (2010) used clustering method for segmenting the TB bacilli. K-means clustering procedure was used to segment the saturation component of CY colour model into TB bacilli and background. Then, median filter and region growing were used to remove unwanted regions.

This paper extends the work by Osman et al. (2010) by analyzing quantitatively several colour components such as RBG, HSI and CY colour models in the clustering process. In order to reduce the processing step, only region growing method is used to eliminate the unwanted regions. The method also introduces a local adaptive thresholding to improve the final segmentation results.

**Theoretical Background:**

The diagnosis of TB infection in tissue is usually depends on the detection of the bacilli in the ZN-stained tissue slide. Colour is the most useful feature that is utilized in detecting the TB bacilli. During the staining process, carbol fuchsin dye is used to colour the TB bacilli red, while the methylene blue turns the tissues and backgrounds to the blue colour. The ZN stain results a good contrast between the bacilli and the background, thus aiding in the detection process. However, the intensity distribution of TB bacilli and background are often varying from image to image, due to manually ZN preparation by technologist. Figure 1 shows examples of ZN-stained tissue slide images which consist of TB bacilli. The bacilli which have a characteristic of red rod-shaped are found scattered irregularly through the tissue images. Some of the bacilli appear stained in deep-red (Figure 1 (a), (c) and (d) and others appear in pale-red (Figure 1 (b)). The improper reagent preparation and staining procedure may also cause the background to remain red (Figure 1 (c) and (d)), thus make the segmentation process more challenging. Therefore a method for TB bacilli segmentation should be robust with respect to these variations.

![Figure 1](image-url)

**Fig. 1:** Example of ZN-stained tissue slide images consisting of TB bacilli. The manually staining slides caused the TB bacilli intensities to vary over different images. The bacilli appear stained in deep-red as in (a), (c) and (d), while in pale-red as in (b). Some background also remained in red colour, indicated by arrows in (c) and (d).

The present study investigates three types of colour space namely the red-green-blue (RGB), the hue-saturation-intensity (HSI) and CY colour spaces for TB bacilli segmentation. The transformation from RGB to HSI and CY are explained in the following.
RGB to HSI Colour Conversion:
The HSI colour model consists of three components, hue (H), saturation (S) and intensity (I). The hue, saturation and intensity represent the colour property, the degree of white light added to pure colour and the brightness of colour, respectively. Consider an RGB image with R, G and B represent their normalized red, green and blue components, respectively. The transformation from RGB to HSI colour space is given as follows (Gonzalez and Woods, 2007).

\[
H = \begin{cases} 
\cos^{-1}\left(\frac{1}{\sqrt{3}}\frac{(R - G) + (R - B)}{(R - G)^2 + (R - B)(G - B)}\right) & \text{if } B \leq G \\
2\pi - \cos^{-1}\left(\frac{1}{\sqrt{3}}\frac{(R - G) + (R - B)}{(R - G)^2 + (R - B)(G - B)}\right) & \text{if } B > G 
\end{cases} 
\]  
(1)

\[
S = 1 - \frac{3}{R + G + B}\left[\min(R,G,B)\right] 
\]  
(2)

\[
I = \frac{1}{3}(R + G + B) 
\]  
(3)

where H, S and I represent the hue, saturation and intensity components of HSI colour model, respectively.

RGB to CY Colour Conversion:
The RGB to CY colour model can be formulated using the following 3×3 transformation (Dougherty, 1999).

\[
\begin{bmatrix} Y \\ R - Y \\ B - Y \end{bmatrix} = \begin{bmatrix} 0.299 & 0.587 & 0.114 \\ 0.701 & -0.587 & -0.114 \\ -0.299 & -0.587 & 0.886 \end{bmatrix} \begin{bmatrix} R \\ G \\ B \end{bmatrix} 
\]  
(4)

where Y, R−Y and B−Y are the luminance, the subtraction of Y from the R component, and the subtraction of Y from B component, respectively. The subtraction of Y from the G component, G−Y is derived from the R−Y and B−Y components as:

\[
G - Y = -0.509 \cdot (R - Y) - 0.194 \cdot (B - Y) 
\]  
(5)

In the CY colour model, the hue, \( \theta \) and saturation, S can also be determined from the R−Y and B−Y components as follows:

\[
\theta = \begin{cases} 
\tan^{-1}\left(\frac{R - Y}{B - Y}\right) & \text{if } S \neq 0 \\
\text{undefined} & S = 0 
\end{cases} 
\]  
(6)

\[
S = \sqrt{(R - Y)^2 + (B - Y)^2} 
\]  
(7)

Methods:
In this work, a fully automated methodology for segmentation of Mycobacterium tuberculosis in tissue sections by combining thresholding and clustering techniques is presented. The segmentation serves as the first stage of a multi-stage procedure for detecting the TB bacilli. The present study employs the global thresholding as presented in Osman et al., (2010) to remove the tissues and background which were stained by methylene blue dye, in order to initiate segmentation of the TB bacilli. For the second step, k-mean clustering is used to extract the TB bacilli from the remaining background. Three types of colour models, the RGB, the HSI and the CY model, are investigated to find the best candidate for the clustering. The third step involves labeling and removing noises and large overstained regions using the automatic seeded region growing (ASRG) method. The study introduces a local adaptive thresholding and a median filter, as a fourth step, to refine the segmented regions. In the following sections, the method will be presented and discussed in detail.
**Tissue and Background Removal:**

The key element of the TB diagnosis is based on the presence of TB bacilli in tissue sections. Although the intensity distribution of TB bacilli and background are often varies from image to image, the bacilli always retain the red colour due to the nature of acid-fast bacilli (AFB) property.

The first step in attempting to segment the TB bacilli in ZN-stained tissue slide images involves removing the tissues and background. The purpose of this step is to find sub-images that contain only pixels with red colour, so that later it will facilitate the process of bacilli extraction. Since the colour property is defined by hue, color segmentation can be performed based on a simple hue space. A global thresholding based on hue space is introduced as follow (Osman, et al., 2010):

$$
P_{\text{g}}(x, y) = \begin{cases} 
P(x, y) & \text{if } 0 < \theta(x, y) < \frac{2\pi}{3} \\
0 & \text{otherwise} 
\end{cases}
$$

where \(P(x, y)\) and \(\theta(x, y)\) are the intensity value in RGB and the hue value at a point \((x, y)\) in an \(x \times y\) image, respectively. An example of applying the global thresholding to a ZN-stained tissue slide image is shown in Figure 2. It can be seen that the process had eliminated most of the tissues and background in the image by removing pixels other than red colour.

![Figure 2](image)

**Tb Bacilli Extraction:**

The second step involves extracting the TB bacilli from the remaining background with the red colour. It is evident from Figure 1 that the intensity distribution of TB bacilli is varying from image to image. It is also observed that most of the TB bacilli have appeared more deep-red compared to the background. Therefore an adaptive solution is required for segmenting the bacilli.

An adaptive pixel segmentation based on k-mean clustering algorithm is implemented on the colour intensities of pixels and grouped the pixels into two clusters; correspond to the TB bacilli and the remaining background. Suppose that \(X = \{x_i \mid i = 1, 2, \ldots, m\}\) is a set of pixels' intensity in an image with \(m\) number of pixels, the k-mean clustering algorithm for image segmentation can be implemented as follows:

1. Choose \(k\) pixels representing the preliminary cluster centroids in an image, \(C = \{c_j \mid j = 1, 2, \ldots, k\}\). Note that \(c_j\) is the intensity level of \(j\)-th centroid.
2. For each pixel, \(x_j\) in an image, calculate the intensity difference between the pixel and the pixels’ centroid, \(d = \|x_j - c_j\|\) (9)
3. Assign the pixel to the cluster with the lowest intensity difference.
4. When all pixels have been assigned, recalculate the new positions of the cluster centroids.
5. Repeat Steps 2 - 4 until the position of the all centroids no longer move.

In this study, the effectiveness of RGB, HSI and CY colour models are investigated. Each component of the colour models is fed to the k-mean clustering and the performance is evaluated. For the CY colour model, the luminance, hue and saturation are chosen for the analysis. Figure 3 shows example of the k-mean clustering results for the three colour models.
Selection of Region of Interest:

Since the segmentation method is solely depends on colour information, some extraneous objects such as small noise particles and overstained backgrounds, having the same colour with the TB bacilli are present, as shown in Figure 3 (b) – (j). In order to remove these undesired small and large regions, automatic seeded region growing (ASRG) method is used to calculate the size of regions (represent by number of pixels) and excludes the undesired regions from the remaining process.

The first processing step is to convert the colour image into a binary image. The process assigns an intensity value of ‘1’ to the object pixel and a value of ‘0’ to the background pixel. Binary image is used for ASRG as it facilitates the automated seed selection and counting the size of regions. Suppose that $P(x,y)$ and $P_b(x,y)$ represent the intensity of a pixel at location $(x,y)$ in a colour image and its binary image, respectively. The procedure for implementing the ASRG is as follows:

1. Scan the binary image pixels. Assign an initial seed to the first pixel associated with an object, $P_b(x,y) = 1$. Assign region label to the pixel.
2. Compare the intensity value of each of an 8-connected-neighbour pixel with the initial seed. Add the neighbouring pixel to a region if it is belongs to object. Assign region label to the neighbouring pixel.
3. Set the neighbouring pixel, which is added to the region, as a new seed location.
4. Repeat step 2 to 3 until the region cannot grow anymore or all the pixels have been considered.
5. Repeat step 1 to 4 for all of the remaining unlabelled pixels that have potential as initial seed location.

Subsequently, the selection of region of interest is determined based on its size. If such a region is supposed to occupy the size within the minimum and maximum of predefined size, $A_{\text{min}}$ and $A_{\text{max}}$ respectively, then the region is preserved by restoring its original intensity of colour pixel, $P(x,y)$. Otherwise, the region will be eliminated from the image. In this work, $A_{\text{min}} = 60$ and $A_{\text{max}} = 800$ were found suitable for the tissue images and were obtained through observations. Figure 4 shows the results of applying ASRG using images from Figure 3, with different colour model components.
Fig. 4: Results of applying ASRG to the k-mean clustering image with (a) red component (b) green component (c) blue component (d) hue of HSI (e) saturation of HSI (f) intensity of HSI (g) hue of CY (h) saturation of CY and (i) luminance of CY.

Region Refinement:

The uneven intensity of TB bacilli, even in the same image, makes the segmentation process difficult. Due to this reason, performing thresholding and clustering based on global image information is occasionally inadequate to segment the TB bacilli properly. Figure 5 (a) and (b) show an original tissue slide image and the result after applying the proposed segmentation method, respectively. The cropped and enlarged regions labeled with black arrow 1 and 2 of Figure 5 (a) are shown in Figure 5 (e) and (i), respectively. It is evident from Figure 5 (f) and (j) that by employing the global segmentation has lead to under-segmentation for a certain regions and cause some background pixels are included in the segmentation results.

Fig. 5: (a) Original tissue slide image and (b) the result after k-mean clustering using the saturation component of CY colour model. (c) The result is further improved by applying local adaptive thresholding and (d) median filter. (e) The first example of a cropped and enlarged region, labeled 1 of Figure 6 (a), (f) the result before and (g) after applying the local adaptive thresholding, and (h) smoothened using median filter (i) The second example of a cropped and enlarged region, labeled 2 of Figure 6 (a), (j) the result before and (k) after applying the local adaptive thresholding, and (l) smoothed using median filter.
In order to overcome the problem, a local adaptive thresholding based on local statistic of a region is proposed. In local adaptive thresholding technique, the aim is to compute a threshold value \( T_n \) for each region in the image. Consider a segmented colour image in which \( P(x,y) \) be the intensity of a pixel at location \((x,y)\) and has \( N \) regions. Also, suppose that an inadequate segmentation of a region is indicated by poor uniformity value of a region. The method starts by calculating the uniformity of each region in the image, as given by:

\[
U_n = 1 - \frac{s_n^2}{\bar{x}_n^2}, \quad n = 1,2,\ldots,N
\]

where \( s_n^2 \) and \( \bar{x}_n \) are the variance and mean intensity of the \( n \)-th region. If a region has a uniformity less than the minimum allowable uniformity value, \( U_{\min} \), then the local adaptive thresholding is applied according to:

The threshold value \( T_n \) is computed using the mean, \( \bar{x}_n \) and standard deviation, \( \sigma_n^2 \) of the region intensity, and is given by:

\[
T_n = \bar{x}_n - k\sigma_n^2, \quad k > 0
\]

where \( k \) is a parameter which controls the value of the thresholding. The value of \( U_{\min} \) and \( k \) are set to 0.95 and 0.80, respectively and are used throughout this paper. The intensity of image \( P(x,y) \) is chosen to be similar to that previously used colour component in the k-mean clustering process. Figure 5 (g) and (k) show the results after applying the local adaptive thresholding. It can be seen that the method yields a better segmentation result by removing the under-staining pixels.

The presence of blurring, non-uniform illumination, over-staining and understaining of tissue slides will result in regions with unsmooth boundary and increase the segmentation error. It is also realized that some features which are sensitive to noise can be quite complicated to describe an object with unsmooth boundary. In order to avoid this problem, a binary median filter is introduced. After trying with different window sizes, a median filter with a neighbourhood of \( 5 \times 5 \) pixels is found suitable for most cases. Figure 5 (g) and (k) show two example of segmented regions before applying the median filter. Figure 5 (h) and (l) show the results after applying the median filter. The filter has successfully smoothed the boundary without altering the region colour information. A summary of the proposed segmentation method is presented in Figure 6.

Fig. 6: Components of the proposed segmentation method.

RESULTS AND DISCUSSION

The fully automatic TB bacilli segmentation method was tested using 10 positive tissue slides. All the slides were prepared by the Pathology Department, Hospital Universiti Sains Malaysia (HUSM), Kelantan, Malaysia. Images of tissue slides were acquired using the Luminera Infinity 2 digital camera mounted on the Nikon Eclipse 80i light microscope. The slides were analysed under 40× magnification. The images were captured by moving the slide to different areas randomly to detect the presence of TB bacilli in tissues. All images are held as 24-bit colour images, in size of 800 pixels × 600 pixels and were stored as bitmap (.bmp) format.

Performance of proposed method with different components of colour models is evaluated by comparing the result of segmented image against a manual-segmentation image. Manual-segmentation images are prepared by manually selecting the TB bacilli and disregarding the unwanted areas using Adobe Photoshop software in. Then, visual assessment of manual-segmentation images will be done by pathologist for validation. Since the manual-segmentation process is tedious and time-consuming, only 100 images are chosen for the manual-segmentation process. However, in order to proof the robustness of the proposed segmentation method, the images are selected so that they consist of various staining conditions such as properly stained, understained and
overstained images. Figure 7 (a) – (c) show some examples of tissue slide images while Figure 7 (d) – (f) are their results of manual-segmentation.

Next, the segmentation performance with each colour components was assessed quantitatively based on the similarity of individual pixels level between the segmented image and the ground truth image. Four statistical measures were adopted in this work; accuracy, sensitivity, specificity and Jaccard coefficient, which are defined as follows:

\[
\text{accuracy} \; (\%) = \frac{TP + TN}{TP + TN + FP + FN} \times 100
\]

\[
\text{sensitivity} \; (\%) = \frac{TP}{TP + FN} \times 100
\]

\[
\text{specificity} \; (\%) = \frac{TN}{TN + FP} \times 100
\]

\[
\text{Jaccard coefficient} = \frac{TP}{TP + FP + FN}
\]

Fig. 7: (a) – (c) Some examples of tissue slide images and (d) – (f) their results after manual-segmentation, respectively.

where \( TP, TN, FP \) and \( FN \) are True Positive, True Negative, False Positive and False Negative pixels, respectively. The accuracy is defined as the percentage of pixels which are correctly classified as TB or background in the image. The sensitivity measures the percentage of correctly classified TB pixels while the sensitivity refers to the percentage of correctly classified background pixels in the image. The Jaccard coefficient measures the degree of similarity and ignores the negative matches. It takes a value of zero if the two sets are least similar, and is closed to 1 if they are identical.

Table 1 tabulates the segmentation performance using the three colour models. The hue component of both HSI and CY colour models were observed to produce less significant difference after applying the k-mean clustering algorithm for most of the segmented images and therefore eliminated from further analyses. The saturation component was the best performing colour component for the segmentation. The component received slightly higher percentage of accuracy, sensitivity and specificity, as well as the Jaccard coefficient, compared to the other colour components. In the saturation space, the TB bacilli usually appear in deep-red and take the higher saturation value while the background with the lower saturation. This makes the process of extracting the bacilli using the saturation component is relatively easy compared to the other components of the colour model. The saturation component derived using the CY colour component offers a more uniform maximum saturation than in the HSI colour model (Dougherty, 1999), thus producing a better segmentation result compared to the HSI.
Table 1: Segmentation performance using different component of RGB, HSI and CY colour models.

<table>
<thead>
<tr>
<th>Colour model</th>
<th>Colour component</th>
<th>Accuracy (%)</th>
<th>Sensitivity (%)</th>
<th>Specificity (%)</th>
<th>Jaccard coefficient</th>
</tr>
</thead>
<tbody>
<tr>
<td>RGB</td>
<td>Red</td>
<td>98.29</td>
<td>44.03</td>
<td>99.06</td>
<td>0.255</td>
</tr>
<tr>
<td></td>
<td>Green</td>
<td>99.17</td>
<td>61.40</td>
<td>99.71</td>
<td>0.498</td>
</tr>
<tr>
<td></td>
<td>Blue</td>
<td>99.19</td>
<td>63.80</td>
<td>99.69</td>
<td>0.486</td>
</tr>
<tr>
<td>HSI</td>
<td>Saturation</td>
<td>99.33</td>
<td>62.56</td>
<td>99.87</td>
<td>0.562</td>
</tr>
<tr>
<td></td>
<td>Intensity</td>
<td>98.97</td>
<td>55.98</td>
<td>99.59</td>
<td>0.417</td>
</tr>
<tr>
<td>CY</td>
<td>Saturation</td>
<td>99.41</td>
<td>81.44</td>
<td>99.71</td>
<td>0.641</td>
</tr>
<tr>
<td></td>
<td>Intensity</td>
<td>99.02</td>
<td>56.42</td>
<td>99.63</td>
<td>0.433</td>
</tr>
</tbody>
</table>

During segmentation, an understained tissue slide has a tendency to miss some pixels belongs to the TB, while for the overstained tissue slides, some background pixels are classified as TB. Both of the understaining and overstaining problems in tissue slide have increased the segmentation error and reduced the percentage of sensitivity for most of the colour components. However, the results of segmentation with the saturation component of CY provided acceptable result with a sensitivity of 81.44%. Figure 8 and 9 show the results of applying the segmentation method to the understained and overstained slide images, respectively. As the method is solely based on the colour information, it can be seen that some unwanted backgrounds, having similar intensities with the TB bacilli, have appeared at the end of the segmentation. Therefore, further research direction will focused on features and shape descriptions that can be used to eliminate these unwanted regions.

Since the amount of TB pixels are relatively small compared to the background pixels in a tissue image, the result of accuracy and specificity produced less significant difference for different type of colour components. The Jaccard coefficient is found to be more suitable compared to the other statistical measures as it ignores the effect of background.

Fig. 8: (a) An example of understained tissue slide image, (b) manual-segmentation of image (a)  and (c) the result after applying the segmentation process using saturation component of CY colour model.

Fig. 9: (a) An example of understained tissue slide image, (b) manual-segmentation of image (a)  and (c) the result after applying the segmentation process using saturation component of CY colour model.

Conclusions:

This paper presented a method for segmenting TB bacilli in ZN-stained tissue slide images. The method exploits and integrates both the thresholding and clustering method to improve the segmentation task. A global thresholding method had been applied to remove the background pixels at the first step and a local thresholding method had been used to refine the boundary region after the clustering process. Three types of colour models, RGB, HSI and CY had been exploited to find the best colour for the k-mean clustering algorithm. The effectiveness of the proposed method had been tested by comparing with the manual-segmentation images. The obtained results indicated that the method produced acceptable result and can be used as first stage of an automated TB bacilli detection for tissue sections.
ACKNOWLEDGMENTS

The authors wish to thank the panel of pathologist from the Department of Pathology, School of Medicine, University Science Malaysia (HUSM), who has participated in this study and permission to carry out work. Special thanks to Che Noriah Che Hak for preparing the tissue slides. Furthermore, the authors are thankful to MOSTI for providing the grant money.

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