Unsupervised Segmentation for Inflammation Detection in Histopathology Images

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Abstract. Acute inflammation of the placenta is associated with an increased rate of perinatal morbidity and mortality. This paper presents a novel method for analyzing digitized images of hematoxylin and eosin (H&E) stained histology slides to detect and quantify inflammatory polymorphonuclear leukocytes (PMLs). The first step is isolating the tissue by removing the background and red blood cells via color thresholding. Next, an iterative threshold technique is used to differentiate tissue from nuclei. Nuclei are further segmented to distinguish between nuclei which match morphological characteristics of PMLs and those which do not, such as connective tissue fibroblasts as well as chorion and amnion epithelium. Quantification of the remaining nuclei, many of which are likely neutrophils, are associated with amniotic fluid proteomic markers of infection and inflammation, as well as histological grading of neutrophils in amnion, chorion and decidua, and in the umbilical cord.

Keywords: Medical image processing, computer aided diagnosis, quantitative image analysis, histopathology image analysis, microscopy images, pathological image analysis, image processing, nuclei morphology.

1 Introduction

The diagnosis of intra-amniotic infection is clinically relevant to perinatal morbidity and mortality. Even among expert pathologists variability in the degree of infection diagnosed occurs with the standard use of qualitative visual examination of hematoxylin and eosin (H&E) stained placenta histology slides [1]. This leads to a less than optimal scoring system to determine the presence of infection, with a significant possibility of contradictory diagnoses within a set of experts examining the same slide.

The goal of this paper is to explore methods to improve diagnostic reliability through automated image analysis. This analysis takes into account known color and shape properties of nuclei related to infection in stained slides. The construction of an automated method for quantification of inflammatory polymorphonuclear leukocytes (PMLs), within the H&E slides would be a novel and useful tool. To address this we are developing a computer aided diagnosis tool

which relies heavily on algorithms that segment and filter out image features that do not fit the criteria of the inflammatory PML nuclei. Inflammatory PMLs are indicative of an intra-amniotic infection. By isolating inflammatory PML cells, quantitative data can be gathered about the degree of inflammation within the tissue sample.

This paper discusses threshold-based segmentation and shape analysis techniques for the purposes of quantification of inflammatory PMLs. Our technique was developed without the assumption that staining techniques can be exactly controlled and replicated consistently across lab environments. We will introduce a technique for removing background information which consists of white backgrounds as well as red blood cells. Our color analysis makes no assumptions about the color channel bit resolution. We will also introduce a novel thresholding technique which is iterative in nature and automates the thresholding process to separate stained cell nuclei from the tissue. Finally the cell nuclei are categorized based on size and shape to determine if they are likely inflammatory PMLs. We believe that providing automated analysis tools based on known physical properties of inflammation indicators in stained slide images is an important step toward increasing the reliability and agreement of expert pathologist diagnoses.

2 Related Work

Much of the work in the literature related to the automated analysis of pathology slides are based on supervised techniques in which manual specification of parameters (such as thresholds and iteration counts) is required by the user. We believe this is detrimental to acceptance of such tools by pathologists with limited (if any) familiarity with the meaning of these parameters and the underlying algorithms. A rich body of literature does exist though related to stained tissue image analysis for diagnostic purposes.

Researchers have proposed analysis methods that increase automation and reduce the burden on the analyst when applying the computational techniques. Wang et al. presented an automated approach to segmentation of cervical tissue but had issues with false positives when their algorithm misclassified red blood cells as their intended target of squamous epithelium [2]. Our proposed method for removing red blood cells prior to applying segmentation techniques solves this problem. Sertel et al. attempted to remove white background pixels to alleviate computational intensity by not analyzing portions of the image with intensity level greater than 200 in all three 8-bit RGB color channels [3]. Upon implementation of this technique we found the fixed value threshold of 200 too low for H&E stained tissue. We present an alternate method which is responsive to image conditions rather than a hard coded value and retains the advantage of reducing the data to pixels which contain tissue rather than background.

Classification using shape and color was used by Kong et al. to differentiate between pathological components of H&E stained tissue [4]. Tosun et al. proposed a shape analysis based on object size to group various tissue components [5]. In section 4.3 we implement a similar approach for differentiating between nuclei types within placental tissue.

At the present, image processing techniques for automated (or even semi-automated) medical image processing is an open and active area of research. There are no publications known to the authors related to image processing for the purposes of identifying inflammatory PML nuclei. The medical methods of diagnosis for placental inflammation are also an open area of research. Attempts at standardized scoring of intra-amniotic infection is still a source of controversy [6]. Salafia et al. argue that the current qualitative approach needs to be improved upon via a reliable quantitative automated tool. The technique presented in this paper is an answer to that call and would allow a more universal and uniform system of diagnosis for the presence of inflammation through the identification of inflammatory PML nuclei [7].

3 Image Dataset

Samples from placental tissue soaked in paraffin and cut at a thickness of $5\mu\text{m}$ are placed on a glass slide. Each slide is prepared using a dual staining process of hematoxylin and eosin (H&E) to create visual contrast between tissue structures. The tissue slide is loaded into an Aperio Scanscope XT and the entire slide is scanned and digitized at $20\times$ magnification with a resolution of $0.5\mu\text{m}$ per pixel.

The image set for developing and verifying this algorithm contained 450 tissue slides with 45 covering variations in H&E stain age and 405 covering variations in H&E stain brand. Stain age was varied on 3 sequential tissue slices from 15 cases and stained at 0, 2, and 4 days old. The brand variation was 8 unique combinations of 4 brands of Haematoxylin and 2 types of Eosin, plus one slice stained with Myelo IHC. This rotation of 9 stain combinations was performed 3 times over 27 sequential tissue slices from 15 tissue samples for a total of 405 slides.

The goal of this variation in brand and stain age was to determine whether or not variations that will be expected in practical laboratory and hospital environments will have a negative impact on the performance and reliability of the algorithm. It is not practical to assume that staining conditions can be exactly replicated across a diverse set of lab environments, therefore a robust diagnostic algorithm must take this into account when evaluating its viability.

4 Methods

The following section will introduce image segmentation for background detection, a novel iterative threshold selection algorithm for cell nuclei detections and shape analysis for isolation of particular nuclei of interest.

4.1 Background Detection (Image Segmentation)

To set the stage for the iterative thresholding technique, the image must first consist of only H&E stained tissue. Removing extraneous features, such as the

white background of the slide and red blood cells, normalizes the image for input into the iterative thresholding technique. The method for removing background and red blood cells makes use of color ratios and is therefore neutral to the number of bits for color representation. Consider the raw image in Figure 1(a) in the following discussion.

Removal of red blood cells is accomplished using a simple threshold. Within an image of H&E stained tissue, any pixel with a red to blue ratio greater than 1.25 is likely too red to be considered tissue or nuclei and can be masked out and ignored. The mask showing the pixels identified as meeting this criteria to be filtered out from Figure 1(a) are shown in Figure 1(b).

Fig. 1. The process of removing red blood cells and white background from an image. The red blood cell mask removes pixels with a red to blue ratio. The background mask removes pixels with a high values in each color channel. Pixels meeting these criteria are represented as black in figure 1(b) and figure 1(c) respectively. Figure 1(d) is the resulting image after masking.

The next step is to remove all background from the image. In the case of slide images, the background is a relative “white” but not necessarily the maximum intensity RGB color value for white. Basing the threshold on a percentage of the maximum value of each channel helps eliminate manual adjustments when a slide is very dark, very light, overcast with excess dye, or the scanner was not calibrated correctly to the background. We can compute the color channel thresholds for determining what we will consider “white” by first identifying the maximum intensity in each color channel: r_{max} , g_{max} , and b_{max} . Given these values, we can select individual thresholds for each channel as:

$$t_{red} = 0.95 \cdot r_{max} ; \quad t_{green} = 0.92 \cdot g_{max} ; \quad t_{blue} = 0.92 \cdot b_{max}$$

The multipliers are intended to select very bright pixels, but not strictly those at the maximum value. This is necessary to take into account variations in the lighting and background due to the imaging process. We choose a slightly stricter threshold for the red channel because we must avoid accidentally confusing sparse tissue (with a pink appearance) with background. Given these thresholds, we are then able to determine if a given pixel should be considered “white.”

$$\text{isWhite}(x) = (x_{red} > t_{red}) \wedge (x_{green} > t_{green}) \wedge (x_{blue} > t_{blue})$$

The mask for background pixels to be filtered out of Figure 1(a) are shown in Figure 1(c). Even when there are no “white” pixels within the image, there is

enough variability among the color channels to push the threshold high enough such that no pixels meet the criteria for thresholding in all three channels (causing at least one clause in `isWhite()` to be false, rendering `isWhite()` to be false), thereby making the method safe for use when no “white” background is present.

4.2 Cell Nuclei Detection (Iterative Threshold)

Once the blood and background are removed from the image, the remaining pixels represent tissue and cell nuclei. A threshold must be established to differentiate tissue from nuclei. The precise value of this threshold is a function of the image contrast due to lighting conditions during imaging and shifts in the mean color intensities due to stain aging and brand variations. To eliminate the need for a technician to dial in the optimal threshold value to account for these variations, an automated method was developed. The iterative technique converges on the threshold for obtaining this distinction between tissue and nuclei with the following method.

Algorithm: Iterative Threshold Selection.

Input: RGB pixel values.

Output: Threshold.

```
threshold  $\leftarrow$  meanIntensity(Pixels)/2
repeat
    for all pixels  $p \in$  Pixels
        if  $p_{red} < threshold$ 
            remove  $p$  from Pixels
            threshold  $\leftarrow$  meanIntensity(Pixels)/2
until nothing is removed from Pixels
return threshold
```

The threshold value returned from the iterative threshold selection algorithm is used to separate pixels belonging to tissue and pixels belonging to nuclei based on a comparison of the red channel value. The red channel is compared because the tissue which appears as shades of pink have a much higher red channel value than the nuclei which are a purple or blue color.

After the threshold selection algorithm was used on the image set, we found the final threshold to be slightly low (dark) for segmenting all of the nuclei in the image due to some nuclei being overlaid with tissue or not as receptive to the dye. We found adding a 10% correction, which is 25 for the 8-bit encoding used by our slide scanner, brought some of the outlier nuclei into view.

4.3 Cell Nuclei of Interest Detection (Shape Analysis)

It is now essential to determine which of the cell nuclei are the nuclei of interest, that is, those of inflammatory PML nuclei. This is achieved via shape analysis. The nuclei of interest are morphologically distinct in size and shape.

The first distinction is that of nuclei size. PML nuclei consistently take on a characteristic size within the image, allowing them to be differentiated from other cell nuclei. Filtering out nuclei that are outside of this range eliminates a large number of non-PML nuclei that have a similar response to the H&E stain. This filtering can be achieved by examining the pixel count of the candidate nuclei.

The second distinction necessary to eliminate non-PML nuclei is that of nuclei shape. PML nuclei have a characteristic circular (or near circular) shape, while other common features of similar size, such as connective tissue fibroblasts, take on a more elliptical and elongated shape. A measure known as the eccentricity can be computed for each feature to perform filtration by shape. A circle has an eccentricity of zero, and increasingly flattened oval shapes have eccentricities that approach 1.0 (Fig 2). Experiments resulted in a distinction between cell nuclei with eccentricity of .95 or above as fibroblasts. The empirical setting was manually verified by an expert of the domain. We made use of Matlab's built in function to calculate eccentricity based on each connected group of pixels.

Fig. 2. Examples of shapes with an eccentricity of 0.00, 0.70 and 0.95 respectively

Inflammatory PML nuclei differ from chorion and amnion epithelium in size. Cell size analysis is dependent on resolution and magnification which prevents the use of empirical values for the range of normal pixel group size but is easily calculated. The size threshold is determined by the following formula:

$$resolution \text{ (pixels per } \mu\text{m}^2) \times cell \text{ size (}\mu\text{m}^2) = pixel \text{ group size.}$$

The threshold range is based on the normal range of cell size. Following the computation of the number of pixels of each connected component, the connected components are eliminated as noise if the number of pixels is below the pixel group size interval, while those that are above the pixel group size interval are eliminated as epithelial cells or larger artifacts. The remaining nuclei are likely belonging to inflammatory PML cells.

5 Results

Although literature exists related to the automated analysis of pathology slides [2–5], the presented approach has the following advantages: (1) Our method accounts for the possibility of red blood cells being present in the image and the impact it could have on causing false positives as was experienced by [2]. Because

Fig. 3. Case study with inflammatory PML nuclei shown in 3(a), fibroblasts in 3(b) and epithelium in 3(c). The mask after thresholding shown in 3(d), 3(e) and 3(f) respectively. Finally 3(g), 3(h) and 3(i) show the results after size and shape filtering.

of the negative impact on results, we preprocess the image to remove red blood cells from the image. (2) Our method for detection and removal of background pixels is responsive to image conditions and independent of bit resolution for RGB encoding as opposed to [3] which presented a hard coded value we found to produce false positives and was dependant on 8-bit RGB encoding. (3) Unlike anything previously published, our technique is for the purpose of detecting inflammatory PMLs.

The effectiveness of our set of algorithms is based on the ability to identify nuclei which are inflammatory PMLs from other tissue features in digitized H&E stained histopathology slides. This has been achieved by first isolating tissue from background and red blood cells then isolating the stained nuclei with the use of automated threshold selection and finally classification by known attributes of inflammatory PMLs. During the development and verification process, a domain-expert visually inspected processed images and was in agreement with the algorithm for isolating nuclei which were likely inflammatory PMLs.

The nuclei identified as inflammatory PML cells are quantified via the number of positive pixels, the percentage of positive pixels and the number of positive pixel groups. *Positive pixel count* is the number of pixels which are identified as likely belonging to inflammatory PMLs. *Percent of positive pixels* is calculated by dividing *Positive pixel count* by the number of pixels that are identified as tissue. The number of *Positive pixel groups* is the number of positive pixel connected components. We implemented our method on an Intel®Core™2 Duo T9300

2.5 GHz processor with 4GB RAM and 32-bit operating system using MatLab R2008b. Execution time ranged from 4.8 to 6.4 seconds for a 2500 x 2500 pixel ROI. This variation is due to the execution time being a function of the number of inflammatory PMLs present in the tissue sample.

The original image in figure 4(a) captures a fetal inflammatory response in the placenta. The results of the segmentation algorithms are shown in figure 4(b) which is a contour map corresponding to the number of pixels representing inflammatory PMLs in a 40×40 neighborhood. The contour lines outline areas with the highest concentration in black and the lowest in white. Quantification of the data in figure 4 indicates the number of positive pixel groups on the right side of the blood vessel is $10\times$ the amount on the left side. This is an indication that the PMLs have migrated from the blood stream to repair injured tissue on the right side of the vessel.

Fig. 4. An example illustrating the original image (a) and the result of our algorithm (b) with black contour lines indicating regions with high inflammatory PML presence

6 Conclusion

Our ongoing work in this area is focused on performing further validation studies with domain experts. These algorithms will be used in a clinical trial conducted by Placental Analytics, LLC to compare our quantification of PMLs to the qualitative grading of inflammation by several expert pathologists as well as the results of amniotic fluid mass resistance (AFMR) scores. AFMR scores have been shown to predict preterm labor due to intra-amniotic infection [8].

An automated method for quantification of inflammatory PMLs could be a useful tool for pathologists determining the severity of amniotic infection. Automated quantification is more reliable in its reproducibility than the current qualitative approach to diagnostics. Reproducible, reliable and unsupervised segmentation for quantification of inflammatory PML cells can be performed via the use of iterative threshold selection and shape analysis.

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