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Segmenting Overlapping Cell Nuclei In Digital Histopathology Images

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Abstract— **Automatic quantification of cell nuclei in immunostained images is highly desired by pathologists in diagnosis. In this paper, we present a new approach for the segmentation of severely clustered overlapping nuclei. The proposed approach first involves applying a combined global and local threshold method to extract foreground regions. In order to segment clustered overlapping nuclei in the foreground regions, seed markers are obtained by utilizing morphological filtering and intensity based region growing. Seeded watershed is then applied and clustered nuclei are separated. As pixels corresponding to stained cellular cytoplasm can be falsely identified as belonging to nuclei, a post processing step identifying positive nuclei pixels is added to eliminate these false pixels. This new approach has been tested on a set of manually labeled Tissue Microarray (TMA) and Whole Slide Images (WSI) colorectal cancers stained for the biomarker P53. Experimental results show that it outperformed currently available state of the art methods in nuclei segmentation.**

I. Introduction

Immunohistochemistry (IHC) is a technique used by Pathologists to test for the presence of clinically important biomarkers in tumours. The management and prognosis of a patient may depend on the level of expression of the biomarker. Currently, interpretation of IHC is performed by experienced pathologists who manually quantify the biomarker expression. However, manually counting is a time consuming and laborious process and in most cases, biomarkers are assessed using semi-quantification methods. These yield poorly reproducible results [1] and thus automated quantification method, which could consistently quantify biomarkers in an objective way, would be desirable.

Many biomarkers are expressed in the nuclei of cells and thus nuclei segmentation is an important step in the automated analysis of IHC. A number of studies have been performed in the automated analysis of nuclei in different kinds of IHC images. Datar et al. [2] used an unsupervised clustering framework for nuclei segmentation in prostate cancer. Jung et al. [3] presented an unsupervised Bayesian classification scheme for nuclei segmentation on mammary invasive ductal carcinomas. Bergeest & Rohr [4] introduced an active contour and level set based global optimization approach for nucleus segmentation in fluorescence images. Very recently, Qi et. al. [5] introduced an algorithm used a single-path voting followed by mean-shift clustering and level set algorithm to separate overlapping cells in breast cancer.

Although these methods produced good results, they became inaccurate when faced with overlapping or densely clustered nuclei. Resolving this problem is still a very challenging task and it arises due to a number of reasons. Firstly, the tissue sections and colour stains are usually unevenly prepared which makes it difficult to separate the nuclei from the cluttered background. Secondly, there always exists a large intensity variation in the nuclei, making it easily to be over-segmented. Thirdly, images of histological sections represent a 2-dimensional view of a 3-dimensional entity. Thus individual nuclei, although in different planes of the section, may appear to be aligned and overlapping when viewed from one perspective. This will cause under-segmentation.

The most common approach to separating overlapped nuclei on IHC images is the watershed algorithm [6]. The traditional watershed algorithm considers regional minimum as starting points may yield over-segmentation. Instead of choosing regional minimum, some authors [7, 8] detected the seeds first and used those seeds as starting points to perform the watershed algorithm. The success of these approaches highly relies upon the accurate detection of these seed points.

To efficiently detect those seed points, Parvin et. al. [9] proposed an iterative voting method which was used to detect the centers of overlapping cells. Al-Kofahi et. al. [10] presented a distance constrained LoG filtering method to detect the seeds. Both of these two methods produced good results. However, as will be shown in the experimental section, these two methods could fail to detect seeds on heavily clustered nuclei in regions with poorly defined borders.

In this paper, we present a multistage watershed based approach for segmenting severely clustered and overlapping nuclei cells while simultaneously trying to reduce both over-segmentation and under-segmentation. We firstly propose a novel method for detecting the initial seeds by utilizing morphological features. Based on these detected seeds, we then present a novel intensity based region growing step coupled with the watershed algorithm to perform the final nuclei segmentation. Our approach is easy to implement, relatively lightweight, but still very efficient. Experiments on a new dataset have shown that our approach outperforms several state-of-art methods in nuclei segmentation.

II. Materials & Method

All experimental work was performed on images of human colorectal cancers which had been stained for the biomarker P53. This is an important marker of mutation of the TP53 gene and is expressed predominantly in the nuclei

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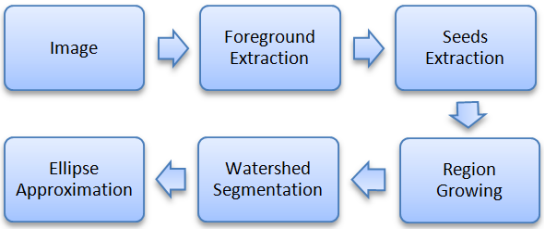

Figure 1. Workflow of the proposed method

of the cells. The IHC was performed using Diaminobenzidine (DAB) as the chromogen. An example of these images is shown in Fig. 2, where the nuclei are severely clustered and there is a large shape variation among different nuclei

Our proposed approach consists of the following three steps. Firstly, the foreground regions which might contain the nuclei are extracted. Secondly, a new seeded watershed algorithm followed by a region growing step is applied to the foreground regions. Thirdly, redundant pixels are eliminated and an ellipse is fit to each segmented nuclei. The whole procedure is also illustrated in Fig. 1.

A. Foreground & Background Classification

In general, the intensity of foreground, i.e. pixels belonging to the nuclei, can be easily distinguished from the background by global threshold method such as Isodata [11]. However, in DAB stained TMA images, the nuclei boundary could be easily misclassified by the global threshold method as the mean intensity levels vary across the background of the entire image. Therefore, global threshold method cannot provide satisfactory results, as shown in Fig. 2(b).

Instead, we used a combination of global threshold and local threshold to tackle this problem. Local thresholding allows an adaptive threshold value to be applied in a local region and can efficiently counter the issue of intensity variations across the background [12]. The local threshold is applied on the pixels which are classified as foreground by global threshold method. We used the same automatic thresholding method [11] for global and local thresholding.

The size of the local thresholding regions (window) and the step-size of the moving window will affect the accuracy of local thresholding results. In order to classify the foreground from the background in a local window, the size of this window should be larger than the size of nuclei in the image. Smaller windows would falsely eliminate the pixels belonging to the nuclei and lead to over-segmentation, while larger windows would cause under-segmentation. Therefore, it is important to set an appropriate size for the local window. The results obtained by different window sizes are shown in Fig. 3(b)-(d). In our experiments, we empirically set it to 50×50.

The horizontal distance that the window moves is determined by the size of the window, which means

$$1 \le d \le s \qquad (1)$$

where d is the horizontal moving distance, and s is the size of the moving window. As shown in Fig. 3, nuclei pixels are largely eliminated when the moving distance is small, especially when d = 1. Although smaller moving

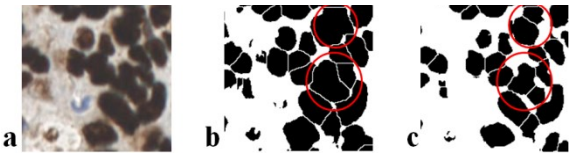

Figure 2. Watershed on extracted foreground regions. (*(a) original image. (b) watershed performed on the global thresholded images. (c) watershed performed on our combined global and local thresholded image.)*

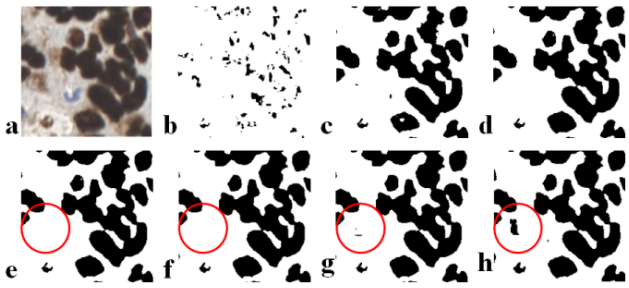

Figure 3. Local window movement. *((a) original image. (b-d) different window sizes, 10×10, 30×30, 50×50. (e-h) different moving distances per iteration, the shown moving distances include 1 pixel, 5 pixels, 15 pixels and 25 pixels.)*

distances (from 1 pixel to 20 pixels) outline the boundary of clustered nuclei very well, weak stained object (circled area) is missed. In all our experiments, we empirically set the moving distance to 25 pixels.

B. Watershed Based On Seeds

Although the combined local threshold and global threshold can clearly detect the boundary of the clustered nuclei, the largely eliminated intra pixels may lead to over-segmentation. Filling the holes offers the potential to minimize the over-segmentation effect. Meanwhile, it may also fill the inter nuclei gaps which may lead to under-segmentation. Thus, the normal watershed may not produce the desired results. Therefore, we prefer to use seeds controlled watershed [7, 8] to segment the clustered nuclei.

Different from previous seeds controlled watershed [7, 8], the seeds used in our approach are obtained through the following two steps. Firstly, two binary masks, mask1 and mask2, are created. The binary mask1 is obtained by the global threshold [11] while the binary mask2 is obtained by the combined global and local threshold method described in section 2.A. The mask2 is then transformed to the Euclidian distance map (EDM) [7]. The initial seeds are obtained by finding the Ultimate Eroded Points (UEP) from this transformed mask, as shown in Fig. 4(a). Watershed splits the overlapped nuclei areas into smaller particles. Instead of focusing on the segmentation accuracy, clustered areas are separated in an over segmentation fashion. Larger sizes of particles are more likely to belong to the nuclei objects, while smaller particles may belong to noise. The removal of noisy particles depends on the mean size range of the nuclei. However, the sizes of nuclei vary across different images, even within a single image. Therefore, a minimum size constraint should be added to prevent over elimination. In the removal process, particles whose sizes are smaller than the mean size or the minimum size constraint are regarded as noise, as shown in Fig. 4(c).

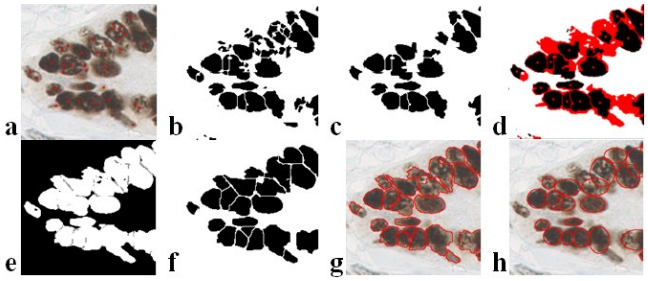

Figure 4. Region growing and nuclei segmentation. *((a) original image, red points in (a) are initial seeds obtained from UEP. (b) the combined local and global thresholded results. (c) small particles in (b) are removed according to the mean nuclei size and minimum size constraint. (d) seeds used for region growing are highlighted in the center of each particle, and the surrounded red pixels are the pixels will be grown. (e) the neck pixels are set to be gray in the region growing process. (f) final segmentation results by watershed. (g) the contour of each nuclei based on (f). (h) ellipses approximation of final segmentation results.*

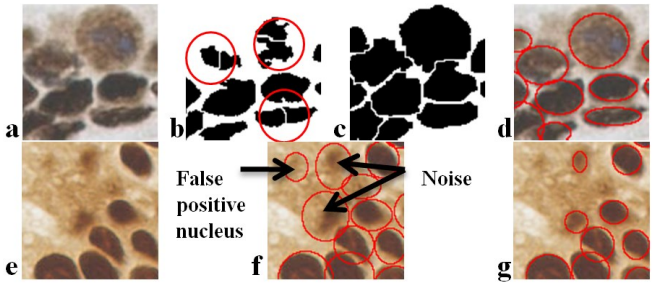

Figure 5. Post processing. *((a) & (e) original image. (b) over-segmented nuclei in the initial segmentation. (c) the merged nuclei after region growing process. (d) final segmentation. (f) falsely identified nuclei before post processing. (g) false positive nuclei are eliminated after post processing.)*

Secondly, the remaining particles in the filtered mask2 are regarded as seeds for the region growing as shown in Fig. 4(d). This region growing process which is based on mask1, aims to retrieve the missed nuclei which are falsely eliminated in mask2 for their weak stain.

This region growing process is similar to the watershed algorithm which floods the water from catchment basins (the seeds) to the dams (the separation lines) where the water comes from different basins meet each other. Different from classical watershed, the cutting "necks" between nuclei are added instead of creating separation dams due to the previous over-segmentation. The "necks" are generated in favor of measuring the intensity of the dam pixels whose intensity is larger than the mean intensity value of that growing nucleus. Adding the "necks" offer watershed the ability to separate the clusters and the potential to merge the over-segmented nuclei as well, see Fig. 5(a)-(d). Based on this neck added binary mask2, the final seeds can be extracted by finding the regional minimal points of EDM. The final segmentation results are generated by the seeds controlled watershed, as illustrated in Fig. 4(f) and (g).

C. Post Processing

As shown in Fig. 5(f), the segmentation results contain noise pixels inside some particles. These noise pixels are usually the stained cytoplasm pixels surround the nuclei.

TABLE I. MEASUREMENT OF SEGMENTATION RESULTS AGAINST MANUALLY LABELED GROUND TRUTH.

	AS	CD	OS	US	Miss	FP	AR	OR
Global[11]	904	664	13	207	381	20	51.5%	52.5%
E-min[13]	925	787	30	78	370	30	61.6%	62.2%
Voting[9]	810	439	74	214	538	83	38.4%	34.9%
LoG[10]	1641	837	338	44	46	422	64.4%	66.2%
Ours	1213	1015	53	75	122	71	81.3%	80.3%

AS=Auto Segmentation, the number of segmented nuclei result. CD=Correct Detection. OS=Over Segmentation. US=Under Segmentation. Miss=Miss segmented nuclei. FP=False Positive. AR=Average accuracy Rate, the average correct detection rate. OR=Overall accuracy Rate, OR=CD/Ground truth.

Therefore, we need a post processing step to eliminate the noise. To achieve this, each particle is examined separately and an intensity histogram is generated for those pixels inside the particle. An auto threshold method [11] is performed on the intensity histogram. Pixels in each particle are considered to be nuclei pixels when the intensity of them is lower than the local threshold. Finally, an ellipse is fitted to those nuclei pixels.

III. EXPERIMENTAL RESULTS

The DAB stained samples dataset includes 52 images of 200x200 pixels. These images contain heavily clustered nuclei areas which are cut from 14 TMA images and 4 WSI of colorectal cancer. We have manually labeled 1265 nuclei in this dataset. Parameters used in this paper are: window size is 50×50 pixels, moving distance is 25 pixels, and the minimum size constraint is 200 pixels. These parameters are empirically decided, although a systemic method to obtain these would be highly desirable, it is still a very challenging task (all data and ImageJ plugin of our method are available at http://www.viplab.cs.nott.ac.uk/download/Nott-Nuclei.html).

We have compared our method with classical watershed performed on global thresholded [11] image and extended minima transformed image (E-min) [13]. Besides, we have also tested iterative voting method presented in [9] by using their publicly available software. The method developed by Al-Kofahi et. al. [10] which has been applied as a toolkit in FARSIGHT [14] has also been compared. For the performance measure, we have adopted different evaluation criterions including correct detection, over segmentation, under segmentation, missing nuclei and false positive. The correct detection means the number of correctly segmented nuclei. The missed nuclei number is calculated as the number of nuclei included in both the under-segmented areas and the foreground areas. False positive nuclei number is calculated as the number of false positive nuclei in over-segmentation areas. Our results together with those obtained by the previous methods are compared in Table 1.

From table 1, we can see that using the global threshold generates more under segmentation and less correct detection. Extended minimal reduces the under segmentation, however, many unevenly stained particles were eliminated and the correct detection number was still low. Iterative voting method locates the center of nuclei based on the border of each nucleus. The weak borders between nuclei in the clustered areas made this method susceptible to under-segmentation. The method used in [10] leads to larger over-segmentation rate than the others due to the highly textured

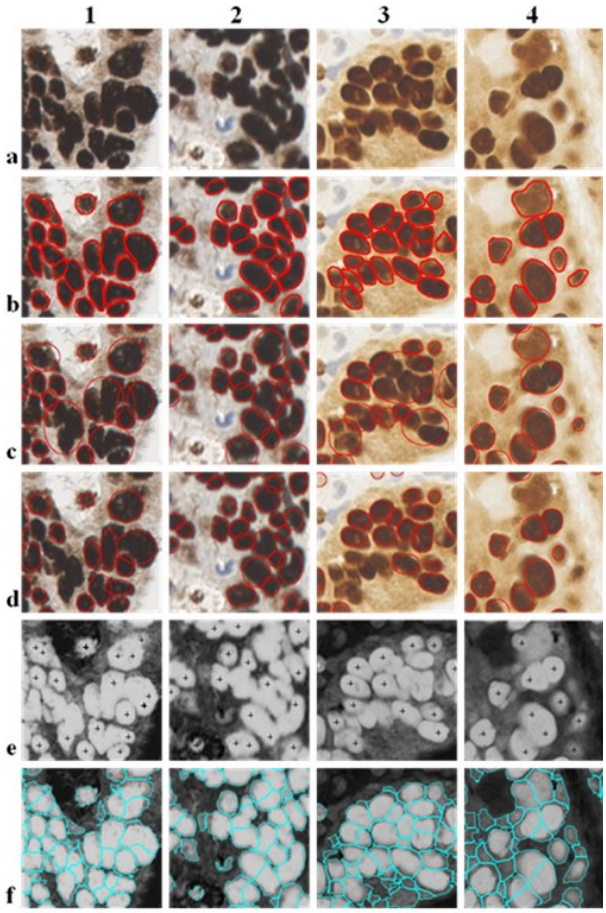

Figure 6. Segmentation results. *(In row (a) original images from 1-4. (b) manually labeled ground truth. (c) watershed on global threshold[11]. (d) watershed on extended minima transform [13]. (e)iterative voting [9]. (f) method in [10].)*

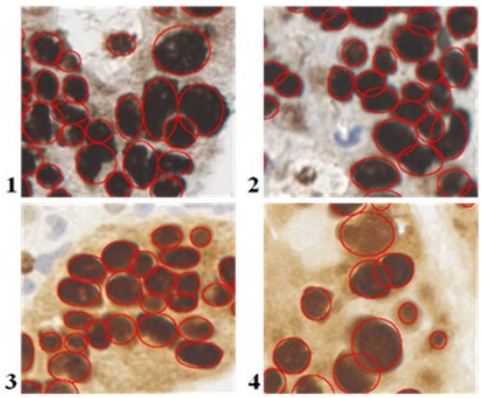

Figure 7. Segmentation results. *(our proposed method.)*

intra chromatin of nucleus and the variations of nuclei structures. Our method balanced the over and under segmentation, significantly increased correct detection and reduced missed detection. Some segmentation results are shown in Fig. 6 and Fig.7.

IV. Conclusion

Quantification of positive nuclei is routinely used in diagnostic pathology for determining individual therapy strategy. In order to reduce the subjective bias of quantification, objective method is of primary importance. In this paper, we have developed an automated method for segmenting clustered nuclei in colorectal cancer. Experiments on a large dataset show that the new method works very well and outperforms several state of the art techniques. As in similar techniques, there are several parameters that need to be determined empirically. Our future work will investigate systematic methods to determine these parameters.

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