Segmentation of Cell Nuclei in Tissue by Combining Seeded Watersheds with Gradient Information

Carolina Wählby and Ewert Bengtsson

Centre for Image Analysis, Uppsala University
Lägerhyddv. 17, SE-75237 Uppsala, Sweden

`carolina@cb.uu.se`
`http://www.cb.uu.se`

Abstract. This paper deals with the segmentation of cell nuclei in tissue. We present a region-based segmentation method where seeds representing object- and background-pixels are created by morphological filtering. The seeds are then used as a starting-point for watershed segmentation of the gradient magnitude of the original image. Over-segmented objects are thereafter merged based on the gradient magnitude between the adjacent objects. The method was tested on a total of 726 cell nuclei in 7 images, and 95% correct segmentation was achieved.

1 Introduction

Automatic image analysis of histo-pathological images, such as fluorescence microscopy images of cell nuclei in tissue, is a non-trivial problem of growing interest. The most difficult step in almost every image analysis task is that of image segmentation, i.e., dividing the image into objects and background as well as separating the individual objects. The difficulty in segmentation of images of cell nuclei in tissue arises mainly due to three things. First, the nuclei are often clustered, touching or overlapping each other. Second, the image background is often uneven due to auto-fluorescence from the tissue and fluorescence from out of focus objects. Thirdly, intensity variations within the nuclei further complicate the segmentation problem.

A method which has proved to be very useful in many areas of image analysis is the so called watershed algorithm, originally suggested by Digabel and Lantuéjoul [3], and then refined and used in very many situations, see [6, 10] for an overview. The watershed algorithm splits the image into regions similar to the drainage regions of a landscape. If the intensity of the image is interpreted as elevation in a landscape, the watershed algorithm can then be used to find mountains, lakes and catchment basins in this landscape.

Cell nuclei can be stained with good contrast using fluorescent nuclear markers. The fairly round shape of the nucleus can be used as an object specific feature in the search for a suitable method for separating clusters of nuclei [1,

2,5,7,11]. This does however require a good method for segmenting the objects and clusters of objects from the background. If the background variation is large, this is not a trivial problem. We here present a method to circumvent the problem of a varying background by segmenting the image based on the gradient magnitude information. If watershed segmentation is applied directly to the gradient magnitude image, it will almost always result in over-segmentation due to the intensity variations within the cells. Some of the over segmentation can be reduced by rule-based merging of the fragmented objects [9,13]. The information about which side of an edge belongs to the inside of an object and which side is outside is not present in the gradient magnitude image. This means that the background may consist of several disconnected objects with different labels, even after merging. The information on what is object and what is background may be extracted from the phase of the gradient, but instead we present a method to solve this, as well as a large part of the over-segmentation, by using morphological filtering to create seeds, i.e., unique identifiers inside each object as well as in the background. Watershed segmentation using seeds has previously been described, for example in [10], but as far as we know, neither followed by merging using in the gradient image nor for this application.

2 Material and Methods

2.1 Cell Preparation and Image Acquisition

The tissue sections used in these experiments come from routinely fixed and paraffin-embedded samples of cervical carcinoma or carcinoma of the prostate. The tissue sections were cut at a thickness of 2-4 μm and deparaffinized and stained as described in [12]. Images were acquired using an epifluorescent microscope from Delta Vision (Applied Precision, Seattle, WA) equipped with a cooled monochrome CCD camera (Photometrics, Tucson, AZ). A Zeiss Plan-Neofluar 63 $\times$ /NA1.30 lens (Carl Zeiss GmbH, Oberkochen, Germany), resulting in a pixel size of 0.1058 μm and an optical resolution of approximately 0.2 μm in the x and y directions.

2.2 Image Segmentation

The segmentation procedure can be described by the steps outlined below. We use the following notation:

I	= input image
I_{seeds}	= extended minima and maxima of I , threshold T_{noise}
$I_{mag(grad)}$	= gradient magnitudes of I
I_{ws}	= watershed segmentation($I_{mag(grad)}$, I_{seeds})
$edgedata$	= edgedata($I_{mag(grad)}$, I_{ws})
I_{result}	= merge(I_{ws} , $edgedata$, threshold $T_{edgedata}$)

Seeding Two kinds of seeds are needed; seeds that represent the different objects in the image (and thus have to be labeled) and a seed for the background, which does not have to be a connected component. Since the objects can be thought of as local maxima and the background contains the minima of the image, the extended maxima and minima transform are suitable for defining the seeds. The extended maxima transform finds the regional maxima of the H-maxima transform. Regional maxima are connected components of pixels with the same intensity value, t , whose external boundary pixels all have a value greater than t [8]. It needs as input T_{noise} , which defines the level of variation allowed within a local maxima. A low T_{noise} value will result in a larger number of seeds. It may result in more than one seed per object. This is however not a major problem since objects with weak borders between them will most likely be merged in the merging step. The extended minima transform is calculated in the corresponding way, using the same T_{noise} value. All pixels detected by the extended minima transform are given the label 1 in the I_{seed} image. The connected components of the extended maxima are labeled from 2 and up. The resulting I_{seed} image can be seen in Fig. 1B.

Calculating the Gradient The seeds of the objects and the seeds of the background should grow and meet where the magnitude of the gradient of the image is at a maximum. There exists many different approximations of the magnitude of the gradient of an image. We have tried several approaches with similar results, and decided to choose the very simple and straight forward Sobel operator [4], consisting of a pair of masks for detection of vertical and horizontal gradients in the image. The weights of the 3×3 mask for detection of vertical lines are (121), (000) and $-1 \times (121)$, and the mask for detection of horizontal lines is the same but rotated 90 degrees. The gradient magnitude image is approximated by the sum of the absolute value of the convolution of the image with the vertical and the horizontal Sobel operator. The result can be seen in Fig. 1C.

Watershed Watershed segmentation can be implemented with sorted pixel lists so that essentially only one pass through the image is required. This implies that the segmentation can be done very fast.

In the paper by Vincent and Soille [11] water rises from all the local minima of the image. The lake created around each minima is referred to as a catchment basin, and the catchment basin continues to grow as water rises, and stops when it reaches a pixel which is located at the same geodesic distance from two different catchment basins. We want to avoid thick watershed lines, leading to pixels that are not part of any catchment basin. In our implementation of the watershed algorithm, we keep track of the pixels that are ambiguous, i.e., located at an equal distance between two catchment basins, and let the water flow around those pixels, leading to much thinner watershed lines than in the original version of the algorithm. The ambiguous pixels are thereafter assigned the most frequent label in its neighborhood, leading to a complete tessellation of the image.

In our seeded version of the watershed segmentation, water can only rise from pixels marked as seeds. Non-seeded minima found in the image are flooded by catchment basins originating from seeds. The water rises until each catchment basin in the gradient magnitude image, i.e., a catchment basin originating either from a foreground or a background seed, meets another catchment basin. Due to the seeding, all catchment basins originating from the background have the same label 1, and can easily be separated from the foreground objects.

Merge Regions with Weak Borders If too many seeds are created in the seeding step, i.e., we have more seeds than there are objects, objects containing more than one seed will be over-segmented. However, if two seeds are in the same object, the magnitude of the gradient at the border between the two parts of the over-segmented object will probably be small, i.e., the border is weak. The strength of the border can be measured in many different ways. A very simple measure is the mean value of the magnitude of the gradient along the border of all touching objects. This is calculated by visiting all border pixels and saving the number of bordering pixels and the sum of the gradient magnitude along the border together with the labels of the two neighbors, resulting in the *edgedata*.

In our application, we are only interested in finding objects that have a relatively large gradient magnitude at their border. A large border gradient magnitude means that the object is well focused, which is important in the further analysis of fluorescent signals from other wavelengths [12]. Therefore, a rather high threshold $T_{edgedata}$ for what to merge is suitable.

3 Results

The algorithm was tested on 7 different images each containing 49-141 cells, 726 cells in total. The result can be seen in Table 1. The same T_{noise} threshold for seeding was used for all images. Depending on $T_{edgedata}$, cells that are badly focused may be lost, as seen in Table 1. The result after a moderate and a high $T_{edgedata}$ is also shown in Fig. 2A and B. The higher percentage of correctly segmented nuclei using the higher $T_{edgedata}$ is partly due to the fact that we discard poorly focused objects as background, i.e., the total number of cells is smaller at the higher threshold.

4 Discussion

The presented method is very simple and straight forward, and probably useful in many different segmentation tasks. No pre-processing is necessary, and only two rather stable thresholds T_{noise} and $T_{edgedata}$ are needed for the full segmentation process. If the $T_{edgedata}$ is set at a high level, objects that are badly focused will be lost, which may be desirable. Further processing, such as removal of cells that are damaged or under-segmented by simple thresholding on shape and size, or more advanced statistical methods, may further improve the result. The method can be extended to more than two dimensions, which is planned as future work.

Fig. 1. **A** The original image; a fluorescence microscopy image of a slice of a tumor. **B** The seeds found by extended minima (gray) and extended maxima (white) transformation. **C** The gradient magnitude image. **D** Result after seeded watershed segmentation. The result is further improved by merging based on edge strength (see Fig. 2A and B).

Fig. 2. **A** Segmentation result after merging at a mean edge gradient amplitude less $T_{edgedata} < 30$. Objects that are a part of the background have now been merged with the background. **B** Segmentation result after merging at a mean edge gradient amplitude $T_{edgedata} < 40$. The over segmented objects are merged, but some of the weakly outlined nuclei have also been merged with the background.

Table 1. Segmentation results (in number of cells)

image	$T_{edgedata} < 30$			$T_{edgedata} < 40$		
	total	over-segm.	under-segm.	total	over-segm.	under-segm.
1	127	3	6	125	1	5
2	90	4	2	56	1	3
3	141	0	5	122	0	2
4	138	0	4	138	0	4
5	92	0	7	92	0	7
6	89	3	3	56	1	3
7	49	0	1	21	0	0
total:	726	10	28	610	3	21
	94.8% correct			96.1% correct		

5 Acknowledgments

Many thanks to Fredrik Erlandsson for providing the images and to Joakim Lindblad and Ida-Maria Sintorn for valuable help and advice.

References

1. J. A. M. Beliën, H. A. H. M. van Ginkel, P. Tekola, L. S. Ploeger, N. M. Poulin, J. P. A. Baak, and P. J. van Diest. Confocal DNA cytometry: A contour-based segmentation algorithm for automated three-dimensional image segmentation. *Cytometry*, 49:12–21, 2002.
2. S. Beucher. The watershed transformation applied to image segmentation. *Scanning Microscopy*, 6:299–314, 1992.
3. S. Beucher and C. Lantuéjoul. Use of watersheds in contour detection. In *International workshop on image processing: Real-time and Motion detection/estimation, Rennes, France*, September 1979.
4. R. C. Gonzales and R. E. Woods. *Digital Image Processing*. Addison-Wesley, Reading, 1993.
5. N. Malpica, C. Ortiz de Solorzano, J. J. Vaquero, A. Santos, I. Vallcorba, J. M. Garcia-Sagredo, and F. del Pozo. Applying watershed algorithms to the segmentation of clustered nuclei. *Cytometry*, 28(4):289–297, 1997.
6. F. Meyer and S. Beucher. Morphological segmentation. *Journal of Visual Comm. and Image Representation*, 1(1):21–46, 1990.
7. P. Ranefall, B. N. L. Egevad, and E. Bengtsson. A new method for segmentation of color images applied to immunohistochemically stained cell nuclei. *Analytical Cellular Pathology*, 15:145–156, 1997.
8. P. Soille. *Morphological Image Analysis: Principles and Applications*. Springer-Verlag, 1999.
9. P. S. Umesh Adiga and B. B. Chaudhuri. An efficient method based on watershed and rule-based merging for segmentation of 3-D histo-pathological images. *Pattern Recognition*, 34:1449–1458, 2001.
10. L. Vincent. Morphological grayscale reconstruction in image analysis: Applications and efficient algorithms. *IEEE Transactions on Image Processing*, 2(2):176–201, 1993.

11. L. Vincent and P. Soille. Watersheds in digital spaces: An efficient algorithm based on immersion simulations. *IEEE Transactions on Pattern Analysis and Machine Intelligence*, 13(6):583–597, 1991.
12. C. Wählby, F. Erlandsson, K. Nyberg, J. Lindblad, A. Zetterberg, and E. Bengtsson. Multiple tissue antigen analysis by sequential immunofluorescence staining and multidimensional image analysis. In *Proceedings of the 12th Scandinavian Conference on Image Analysis (SCIA)*, pages 25–31, Bergen, Norway, June 2001.
13. C. Wählby, J. Lindblad, M. Vondrus, E. Bengtsson, and L. Björkesten. Algorithms for cytoplasm segmentation of fluorescence labelled cells. *Analytical Cellular Pathology*, 24:101–111, 2002.