Maximum-Intensity-Linking for Segmentation of Fluorescence-Stained Cells

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***Abstract*— Fluorescence microscopy is a key technology for the development of new agents in the area of pharmaceutic biotechnology and an important element of computer aided diagnosis and prognosis of tumor cells. A critical component of automatic image analysis methods for cell screening applications is cell segmentation. That is, in order to determine any specific cell features, the individual cells have to be segmented from each other and the background. We propose an approach called 'maximum-intensity-linking' for the segmentation of fluorescence stained cells that is related to classic watershed methods. It is simple, robust and general enough to be easily adapted to changing segmentation requirements.**

I. Introduction

The microscopic image acquisition of fluorescence stained cells under UV-light is a key technology for the development and evaluation of new agents in the area of pharmaceutical biotechnology. Furthermore, it is an important element in the context of new diagnostic methods for the early diagnosis and prognosis of tumors, since fluorescence microscopy enables the exact detection and localization of proteins as well as the visualization of intra-cellular procedures. Today there is a high demand for fast evaluation methods which allow the determination of the expression profile of individual cells. A similar demand exists in the area of early diagnosis and prognosis of tumor cells using fluorescence markers. In order to determine any specific cell features such as size, area, circumference or mean intensity, all individual cells have to be separated (segmented) from each other and the background in a first step.

For the automatic segmentation of cells in micrographs, many well-understood methods have been developed in the past 30 years, cf. Sec. II. Nevertheless, as a required prerequisite most of these methods assume distinct borders between the cells and the image background as well as between neighbouring cells. In contrast to pure morphological stainings as the Papanicolaous stain or the MGG stain which enhance morphological cell structures (plasma or nucleus), this is not the case with fluorescence stainings, where such good-natured preconditions cannot be found. Thus, fast and robust segmentation of irregular shaped and bad-bordered cells in fluorescence micrographs is still an open challenge. Hence, in this work we propose an approach called 'maximum-intensity-linking' for the segmentation of fluorescence stained cells. Our approach is simple, robust and general enough to be easily adapted to changing segmentation requirements.

This work is organized as follows: In Sec. II, a short summary of the state of the art will be given, whereas in Sec. III our new method will be described in detail. Sec. IV describes experiments and results yielded by this method, which will be summarized and discussed in Sec. V.

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II. State of the Art

Many different approaches addressing the problem of cell segmentation from microscopy images have been published in the past 30 years. Most of these works concentrate on the segmentation of morphological structures such as the cell nuclei [1], [2] or the complete cell (nucleus and plasm) [3].

Anoraganingrum et al. [4] apply region growing and adaptive segmentation methods to cell localization and segmentation. Wu [5] et al. apply a two-step method for coarse and fine segmentation using hierarchical thresholding. Pham et al. [6] apply Otsu's well known thresholding method and adaptive fuzzy c-means clustering to fluorescence microscopy image segmentation. Alternatively, watershed algorithms [7], [8], [9] have been tested by several working groups for cell detection and segmentation of cell plasmas and nuclei, as well as active contours for the segmentation of nuclei [10]. Our approach is conceptually related to these classic watershed approaches.

One of the most promising approaches to the separation of neighbouring cells is mathematical morphology. A recent example for this methods is the work by Metzler et al. [11], who make efficient use of a morphological multi-scale approach to separate *touching* cells in binarized images.

Fernandez et al. [1] and Jarkrans [2] both try to detect and separate directly neighbouring cell nuclei. Fernandez et al. propose the detection of *dominant* or *concave* points on the binary contour of the region as points where a splitting of the nuclei might be possible. In contrast, Jarkrans uses a contour analysis based on a smoothed chain code, which reflects the curvature of the nucleus contour.

Wählby [8] recently proposed a multi-step algorithm for the segmentation of cells in fluorescence images. After an initial segmentation using a watershed algorithm, small regions are either merged or deleted. This method is able to split cell aggregates into cell-like regions, but does not separate or treat any overlaps between these cells.

Nattkemper et al. [12] use a neural network approach for the segmentation of fluorescence lymphocyte cells in tonsil tissue. However, their approach is specialized to a certain fluorescence microscopy technique which emphasises the cell contours.

III. Method

The algorithm can be divided into three steps. A preprocessing step smoothes the fluorescence microscopy image and

separates the foreground (cells) from the background. Afterwards individual cells are segmented from each other using our novel region segmentation algorithm called 'maximum-intensity-linking'. Finally, oversegmentations (i.e. cells which are spuriously split into multiple regions) are automatically merged in a postprocessing step.

A. Preprocessing

The intensity of a pixel in fluorescence microscopy images is proportional to the amount of fluorescence marker that is present at the corresponding location in the observed specimen. Even if there is no fluorescence marker at a certain location, the corresponding pixel will not be totally black because of scattered light and sensor noise. Hence the foreground (cells) needs to be separated from the irrelevant background before individual cells can be separated from each other. We use a thresholding operation and determine the threshold automatically based on a black-image acquired using the same acquisit (aperture, exposure time, etc.) that will be used for processing specimens later on. As our region segmentation algorithm performs best on smooth images, we apply a Gaussian low-pass filter to the thresholded image in a second preprocessing step.

B. Maximum-Intensity-Linking

Maximum-intensity-linking is based on the idea to represent an image as a directed graph structure. In this graph structure each pixel is a node that is linked to the pixel in its immediate (8-connected) neighbourhood with the largest intensity value, larger than the intensity of the pixel itself. Pixels that do not have neighbours larger than themselves do not link to anything. The resulting directed graph is a set of trees, which have local maxima as their roots. Once this graph structure is established the algorithm assigns a region-id to each separate tree. This approach results in one region for each local maximum. Hence noise in the form of local peaks leads to oversegmented results, which is the reason for the Gaussian smoothing step. Fig. 1 shows a zoomed detail of a typical fluorescence microscopy image of L929 fibroblasts. A visualization of the maximum-linkage directions for this image is shown in Fig. 2.

It becomes apparent why this algorithm is well suited for segmenting the rather blurry images resulting from fluorescence microscopy by examining the intrinsic meaning of linking each pixel to its largest neighbour (maximum-linkage). The vector from one pixel to its maximum neighbour is, especially after the presmoothing step, a rough discrete approximation of the local image gradient. Therefore, the algorithm traces the gradient until it reaches a local maximum. This discrete approximation has the advantage that it is predictable and deterministic which allows to build a well defined graph structure on top of it.

Regarding maximum-linkage as an approximation of the gradient also explains how the region boundaries are formed. The region boundaries are strongly related to the zero crossings of the (approximated) image gradient. The advantage is that there is no need to generate closed areas based on these zero crossings as region boundaries, as it would be necessary if the algorithm worked explicitly with the extraction of the gradients zero crossings for segmentation.

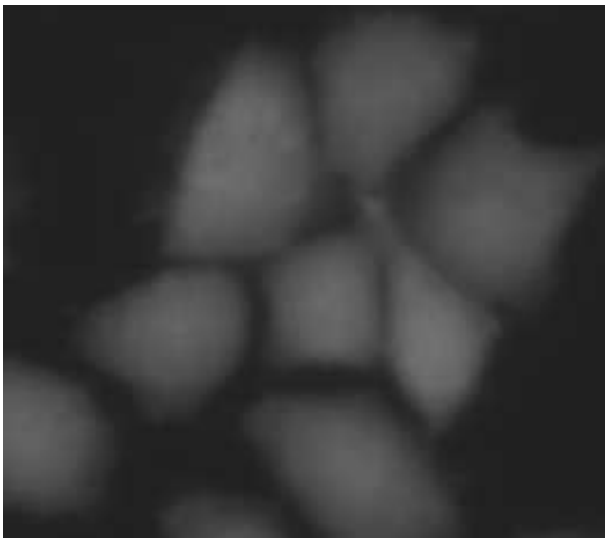

Fig. 1. A zoomed detail of a typical low contrast fluorescence microscopy image, of L929 fibroblasts.

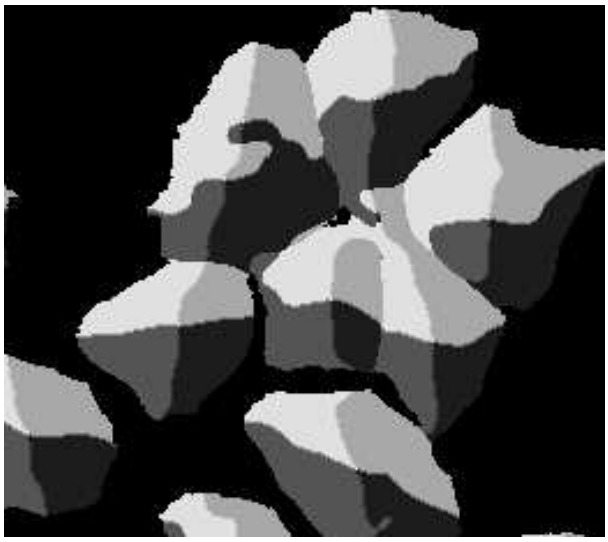

Fig. 2. An intensity-value coded visualization of the maximum-linkage directions for the fluorescence microscopy image shown in Fig. 1.

In practice it is not necessary to explicitly build up the complete graph structure, since the labeling can be done on the fly during the build process. In detail, the region linking algorithm works as follows: Regions are defined by unique identifiers which are assigned to each pixel of the image. For each pixel of the input image, a path of linked pixels along the tree is found by recursively stepping in the direction of the largest local neighbour:

- If a pixel encountered on the path does not have an identifier yet and is not a local maximum, it is stored in a list for later reference and the path is followed by advancing in the maximum-linkage direction.
- If a pixel that is encountered on the path does not

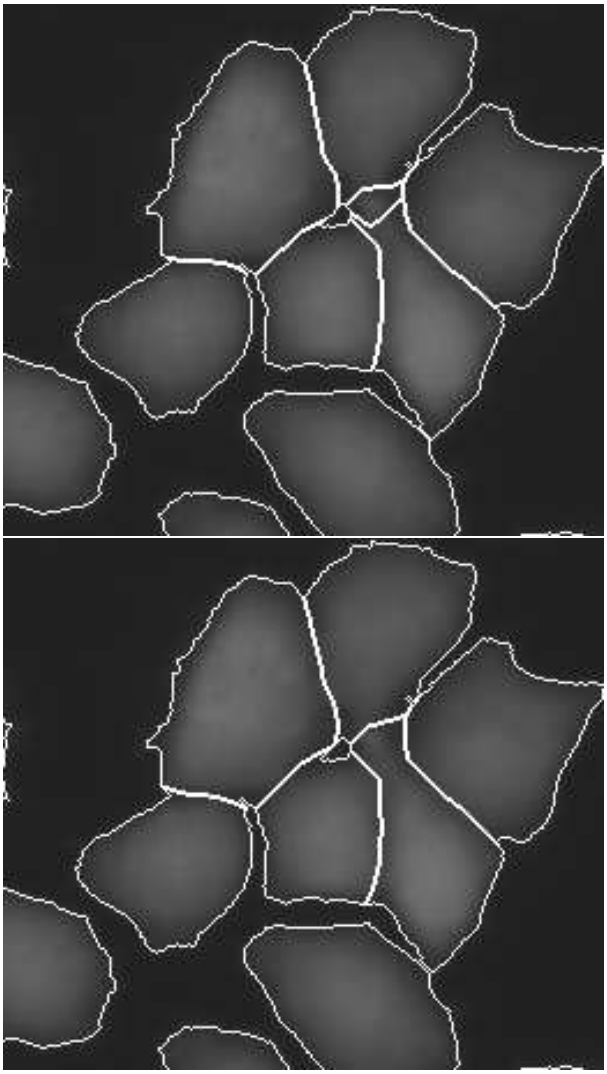

Fig. 3. Segmented regions of the fluorescence microscopy image shown in Fig. 1 for different standard deviations ($\sigma = 4.0$ on the top and $\sigma = 6.0$ on the bottom) of the Gaussian filter. Strong filtering results in less local intensity maxima which ultimately results in less oversegmentation.

have an identifier yet and is a local maximum, a new unique identifier is initialized and assigned to all pixels encountered on the path so far, thereby defining a new region.

- If a pixel that already has an identifier is encountered during this operation, an already processed subtree has been found. Hence, there is no need to follow the path all the way up to the root of the tree. Instead, all pixels on the path so far are assigned the identifier of the encountered tree, therefore making this path part of the encountered tree and its region.

Using this simple algorithm, an image can be segmented into regions in linear time. The number of regions that are found depends on the number of local intensity maxima, which in turn depends on how strong the input image was smoothed by Gaussian filtering in the preprocessing step. Fig. 3 shows the resulting regions for varying standard deviations of the Gaussian filter. In practice, we use rather light smoothing.

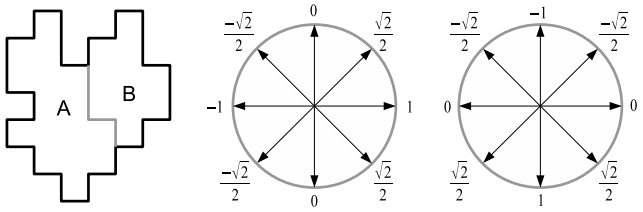

Fig. 4. Due to the discrete pixel representation of images, a boundary (gray) between two regions consists of horizontal and vertical steps (left). Maximum-linkage can be represented as normalized direction vectors. The x- and y-axis components of the direction vectors read as shown on the left and on the right respectively.

C. Postprocessing

Region segmentation using maximum-intensity-linking usually results in slightly oversegmented images. In practice we have found that approximately 15% of the cells in an image are oversegmented on average. However, this problem can be automatically fixed by merging regions based on a criterion derived from the maximum-linkage directions of pixels on the boundary of two regions. If these directions are explicitly written as normalized vectors, the x- and y-axis components of these vectors are defined as shown in Fig. 4.

Due to the discrete pixel representation of images, a boundary between two regions consists of horizontal and vertical steps. Considering a single horizontal boundary step and the direction vectors l and r of the pixels left and right of the boundary step respectively, we define the weight of the horizontal step as $(l_x - r_x)^2/4$. Equivalently the weight of a vertical boundary step is defined considering the direction vectors t and b of the pixels on top and below the boundary step respectively as $(t_y - b_y)^2/4$. If the average of these weights along a boundary between two regions is higher than a threshold (we usually use 0.1), the two regions are considered as an oversegmentation of a single cell and are merged. Fig. 5 shows region boundaries and the intensity coded weights. Both regions which represent an oversegmentation of a single cell and thus should be merged and also regions which represent different cells and therefore should not be merged are shown. In a few cases this criterion is not sufficient and regions which do not represent an oversegmentation of a single cell are merged. We compensate this problem using a heuristic to assure that two large regions which are connected by a very short boundary are never merged. In more detail, we do not merge regions if the length of their common boundary is less than 10% of the length of each of their individual boundaries.

IV. Experiments and Results

We have evaluated our region segmentation algorithm on 62 fluorescence microscopy images acquired using different acquisition parameters and using different types of specimens (amongst others L929 fibroblasts and HeLa cells). The images have a resolution of 1376×1032 pixels and contain 150 cells on average. On a 3Ghz Pentium 4 machine, the processing time is approximately half a second per image. Most of the images are difficult to segment because of low contrast, noise and touching as well as overlapping cells. A manual annotation

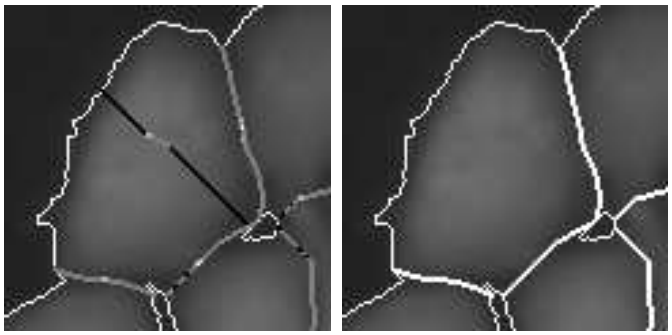

Fig. 5. In a zoomed detail (left) of a segmentation result the weights of the boundary steps for boundaries between each two regions are displayed using intensity encoding. High boundary weights are represented by light, low weights by dark colors. Based on the averaged weights of the boundaries between each two regions, oversegmented regions are automatically merged and valid boundaries are kept (right).

Fig. 6. Many of the images used for evaluation have low contrast and contain cell clusters with poor cell boundaries (left). A semiautomatic (livewire) segmentation done by an expert is a poor reference for the exact cell boundaries (middle) and hence is not used for the evaluation of the boundaries resulting from our automatic segmentation (right).

of the images done by an expert suggests that there are a total of 9387 individual cells. Our automatic segmentation algorithm correctly identifies 9185 individual cells which is equivalent to $97,8\%$. The 202 errors result from 42 over- and 160 undersegmentations, which is equivalent to $0,5\%$ and $1,7\%$ respectively. These numbers suggest that our algorithm tends to under- more often than to over-segmentations. Hence, we plan to add an additional heuristic to solve some of the under-segmentations in the future. The standard deviations of the number of over- and undersegmentation errors across all images are $\sigma = 2,6$ and $\sigma = 0,7$ respectively. This indicates the robustness of our approach to different acquisition parameters and specimens.

Besides the correct number of cells, we are also interested in the quality of the cell borders that are found by our algorithm. However, many of the cells in the evaluation images are irregular shaped and bad-bordered. While the manual segmentation results, which are used for the evaluation, are very exact with respect to the number and location of individual cells, they are not suitable for a qualitative evaluation of the cell borders due to the hard to decide exact cell boundaries (see Fig. 6). We believe that a meaningful evaluation of the segmented cell boundaries is not possible if manually-segmented data is used as a reference. Therefore we plan to use simulated images based on a parametric model like the one proposed by Lehmussola et al. [13] to generate exact reference data in the future.

V. Conclusion

We have proposed a novel algorithm for the segmentation of cells in fluorescence microscopy images. The algorithm is based on a concept we call 'maximum-intensity-linking', closely related to classic watershed methods. The algorithm is both simple and fast. All steps have linear time complexity. In contrast to related watershed methods it is less complex and the postprocessing step for merging of oversegmented regions is based on an objective metric induced by the main algorithm. The evaluation of a large number of images acquired using different acquisition parameters and different types of specimens proves the accuracy and robustness of our approach. Our approach is general enough to be easily adapted to changing segmentation requirements and is already employed in a prototype system which automatically determines the reaction kinetics and the expression profile of individual cells in fluorescence microscopy specimens.

References

[1] Gabriel Fernandez, Jean Paul Zyrd, and Murat Kunt, "A New Plant Cell Image Segmentation Algorithm," in *Image Analysis and Processing, 8th International Conference, ICIAP, San Remo, Italy, September 13-15, 1995*. 1995, vol. 974 of *LNCS*, pp. 229 – 234, Springer.

[2] T. Jarkrans, *Algorithms for Cell Image Analysis in Cytology and Pathology*, Phd-thesis, Faculty of Science and Technology, Uppsala University, Sweden, 1996.

[3] Jens Byriel, "Neuro-Fuzzy Classification of Cells in Cervical Smears," Masters Thesis, Technical University of Denmark, Lyngby, February 1999.

[4] Dwi Anoraganingrum, "Cell image segmentation: Global vs. local adaptive segmentation," in *Proceedings of ISSM Kassel, Germany*, October 1999.

[5] K. Wu, D. Gauthier, and M. Levine, "Live cell image segmentation," Tech. Rep., Center for Intelligent Machines, McGill University, Montreal, Kanada, October 1995.

[6] Tuan D. Pham, Denis I. Crane, Tuan H. Tran, and Tam H. Nguyen, "Extraction of fluorescent cell puncta by adaptive fuzzy segmentation," *Bioinformatics*, vol. 20, no. 14, pp. 2189–2196, 2004.

[7] Pascal Bamford and Brian Lovell, "A Water Immersion Algorithm for Cytological Image Segmentation," in *Proceeding of Segment '96*, Sydney, December 1996.

[8] Carolina Wählby, Joakim Lindblad, Mikael Vondrus, Ewert Bengtsson, and Lennart Björksten, "Algorithm for cytoplasm segmentation of fluorescence labelled cells," *Analytical Cellular Pathology*, vol. 24, pp. 101 – 111, 2002.

[9] Olivier Lezoray and Hubert Cardot, "Cooperation of color pixel classification schemes and color watershed: a study for microscopical images," *IEEE Transactions on Image Processing*, vol. 11, no. 7, pp. 783–789, 2002.

[10] V. Meas-Yedid, Florence Cloppet, A. Roumier, A. Alcover, J-C Olivo-Marin, and G. Stamon, "Quantitative microscopic image analysis by active contours," in *Proc' IAPR-Vision Interface, Ottawa, Canada*, 2001, pp. 277–284.

[11] Volker Metzler, Thomas Lehmann, Hans Bienert, Khosrow Mottaghy, and Klaus Spitzer, "Scale-independent shape analysis for quantitative cytology using mathematical morphology," *Computers in Medicine and Biology*, vol. 30, pp. 135–151, 2000.

[12] Tim W. Nattkemper, Heiko Wersing, Walter Schubert, and Helge Ritter, "A neural network architecture for automatic segmentation of fluorescence micrographs," in *ESANN*, 2000, pp. 177–182.

[13] Antti Lehmussola, Jyrki Selinummi, Pekka Ruusuvuori, Antti Niemisö, and Olli Yli-Harja, "Simulating fluorescent microscope images of cell populations," in *27th Annual International Conference of the IEEE Engineering in Medicine and Biology Society (EMBC'05)*, 2005.