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Orientation Based Segmentation for Phase-Contrast Microscopic Image of Confluent Cell

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***Abstract*— In this research, we propose a novel segmentation method for image of cultured cell at a confluent state, obtained by phase-contrast microscope, based on the orientation. First, we assign to each pixel in the image the direction of an eigenvector corresponding to a smaller eigenvalue of the 2 by 2 Hessian matrix with respect to brightness. Next, we define the orientation at a certain pixel as the histograms of the direction at pixels in the surrounding regions. Then, we evaluate deviation of histograms in the individual regions by entropy, and regard the series of entropy as a multi-dimensional vector, the dimension of which corresponds with the number of regions. We suppose that the vector is assigned to the pixel of interest. Finally, we segment the image based on the multi-dimensional vector using *K*-means method. We investigate the efficacy of the proposed method using an actual human confluent fibroblast image acquired by phase-contrast microscopy.**

I. Introduction

It is important to monitor the state of cultured cells for the production of stable and safe cells for regenerative medicine. Presently, the quality control of the cultured cells depends only on empirical judgment of the cell culture experts using the visual information from the microscopy. Therefore, the effective image processing technique to automatically and objectively analyze the cellular condition is now strongly required.

Cells grow in vitro reaches the state of confluent after proliferation. This is the state to be checked for daily continuous passage culture. Many differentiation induction processes are carried out in the confluent state. However, it has been extremely difficult to segment confluent cells into individual cells because of the lack of morphological features among cells, and difficulties in setting the ground truth for the image as a result of too tight cellular contacts. Therefore image-based analysis of this stage has been extremely rare in the field of cellular biology.

We are proposing a novel segmentation algorithm for the image of cultured cells at a confluent state, where the image is segmented into regions, each of which is homogeneous in the orientation. We investigate the efficacy of the proposed method by applying it to an actual confluent cultured cell image acquired by phase-contrast microscopy. As the model image, we have used normal human fibroblasts at the confluent state obtained by phase-contrast microscopy as shown in Fig.1 (pixel size: 1360 × 1024, gradation: 12 bit). Phase-contrast microscopy is an optical microscopy based on illumination technique that converts phase shifts due to the variance of refractive index in light passing through a transparent specimen to brightness changes in the image, which is widely used to observe cell growth process in a non-invasive manner. In Fig. 1, it is observed that cells with gray brightness adhere to each other, each of which is surrounded by brighter regions so called halo. While orientations are recognized in the figure, the orientations depend on position. The cells are oriented in various directions, and we observe that the region where the directions are in a chaos (regions A), the region whose direction is one way (region B), and the region where the two directions join (region C) are intermingled together.

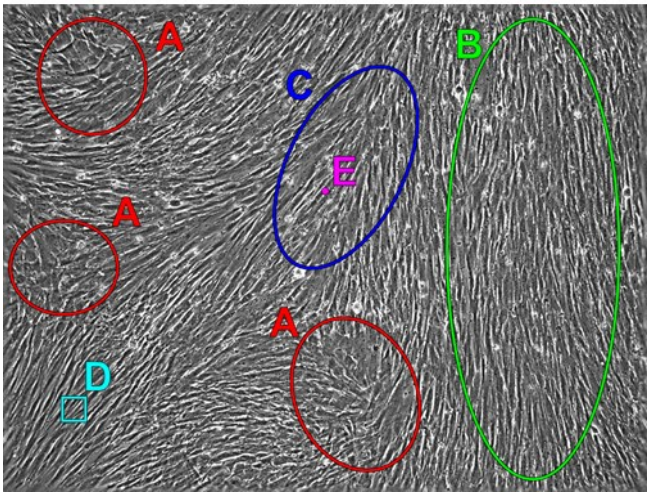

Figure 1. Phase-contrast microscopic image of normal human fibroblasts in the confluent state.

II. Segmentation Algorithm

A proposed segmentation algorithm is as follows: (1) We assign to each pixel in the image the direction of an eigenvector corresponding to a smaller eigenvalue of the 2 by 2 Hessian matrix with respect to brightness. (2) We define the orientation at a certain pixel as the histograms of the direction at pixels in the surrounding regions. Then, we evaluate deviation of histogram in the individual regions by entropy, and regard the series of entropy as a multi-dimensional vector, the dimension of which corresponds with the number of regions. We suppose that the vector is assigned to the pixel of interest. (3) We segment the image based on the multi-dimensional vector using *K*-means method. Below, we describe each processing in detail.

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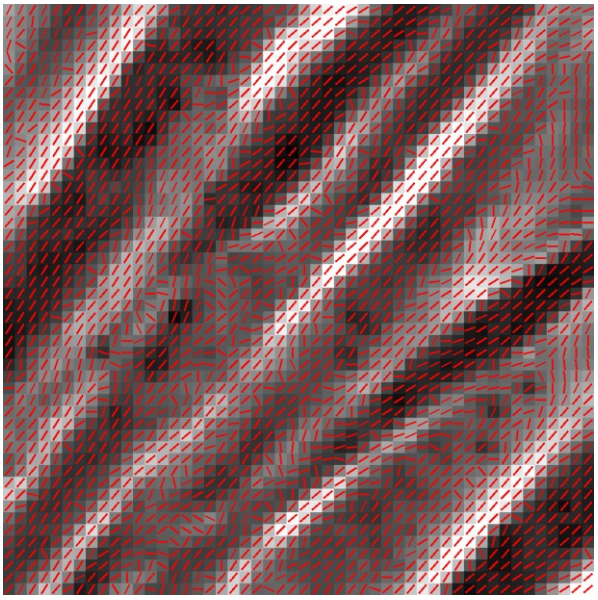

Figure 2. Orientation angle assigned to each pixel in region D of Fig. 1 using the proposed algorithm, where each red segment at the pixel is drawn along the direction of eigenvector $\boldsymbol{e}_2$.

A. *Assignment of direction*

Let $I(\boldsymbol{x})$ be a two-dimensional grayscale image, where $\boldsymbol{x} = (x, y)^t$. The Hessian is defined as

$$\nabla^2 I(\boldsymbol{x}) = \begin{bmatrix} I_{xx}(\boldsymbol{x}) & I_{xy}(\boldsymbol{x}) \\ I_{yx}(\boldsymbol{x}) & I_{yy}(\boldsymbol{x}) \end{bmatrix}, \quad (1)$$

where $I_{xx}(\boldsymbol{x}) = \frac{\partial^2}{\partial x^2} I(\boldsymbol{x})$, $I_{xy}(\boldsymbol{x}) = I_{yx}(\boldsymbol{x}) = \frac{\partial^2}{\partial x \partial y} I(\boldsymbol{x})$, and $I_{yy}(\boldsymbol{x}) = \frac{\partial^2}{\partial y^2} I(\boldsymbol{x})$.

Let λ_1, λ_2 $(|\lambda_1| \geq |\lambda_2|)$ be the eigenvalues of the Hessian $\nabla^2 I(\boldsymbol{x})$, and let $\boldsymbol{e}_1$, $\boldsymbol{e}_2$ be the corresponding eigenvectors. It is known that the Hessian gives information on a local second-order structure in the neighborhood of a pixel of interest [1, 2]. The local structure in a pixel of interest is closely related to the eigenvalues of the Hessian. That is, $|\lambda_1|$, and $|\lambda_2|$ represent the absolute curvature in directions $\boldsymbol{e}_1$, and $\boldsymbol{e}_2$ at point $\boldsymbol{x}$, respectively. Therefore, if we want to know the direction in which the sub-image in the neighborhood of point $\boldsymbol{x}$ stretches, all we have to do is to obtain $\boldsymbol{e}_2$, whose absolute eigenvalue is smaller, after performing eigenvalue decomposition with respective to the Hessian at point $\boldsymbol{x}$. When $\boldsymbol{e}_2 = (e_x, e_y)^t$ is obtained at point $\boldsymbol{x}$, the orientation angle is defined as

$$\theta = \tan^{-1} \frac{e_y}{e_x}, \quad (2)$$

where $-\frac{\pi}{2} \leq \theta < \frac{\pi}{2}$.

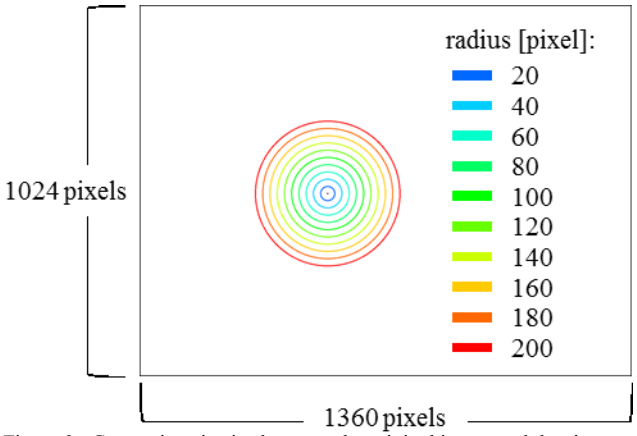

Figure 3. Comparison in size between the original image and the ring regions.

The elements of the Hessian were actually calculated as follows:

$$I_{xx}(\boldsymbol{x}) = \frac{\partial^2}{\partial x^2} G(\boldsymbol{x}) * I(\boldsymbol{x}), \quad (3)$$

$$I_{xy}(\boldsymbol{x}) = I_{yx}(\boldsymbol{x}) = \frac{\partial^2}{\partial x \partial y} G(\boldsymbol{x}) * I(\boldsymbol{x}), \text{ and} \quad (4)$$

$$I_{yy}(\boldsymbol{x}) = \frac{\partial^2}{\partial y^2} G(\boldsymbol{x}) * I(\boldsymbol{x}), \quad (5)$$

where $G(\boldsymbol{x})$ is Gaussian function with a standard deviation of σ, and * means convolution. The standard deviation σ should be on a similar order of the orientation structure observed in size. So, we set it to the value nearly equal to the approximate width of cells.

The processing was applied to a 50 × 50 sub-image surrounded by a square D in Fig. 1, where the cells are highly oriented. In Fig. 2, the direction of each pixel assigned is shown as the red segment on the original magnified sub-image. From the figure, although some segments are oriented in directions different from those of cell orientation, they seem to broadly reflect the cell orientation.

B. *Histogram of directions and entropy map*

As seen in the preceding section, the orientation angles broadly represent the cell orientations. However, some are oriented in directions different from the cell orientation, which are recognized as noise. Therefore, to straightforwardly recognize the orientation angle as the cell orientation will lead to low-precision analysis. Then, we define an orientation of a pixel of interest as the histogram about direction at pixels in the surrounding regions around the pixel of interest. Furthermore, cell orientation will depend on the distance from the pixel of interest. So, we prepare the regions according to the distance from the pixel of interest as shown in Fig. 3.

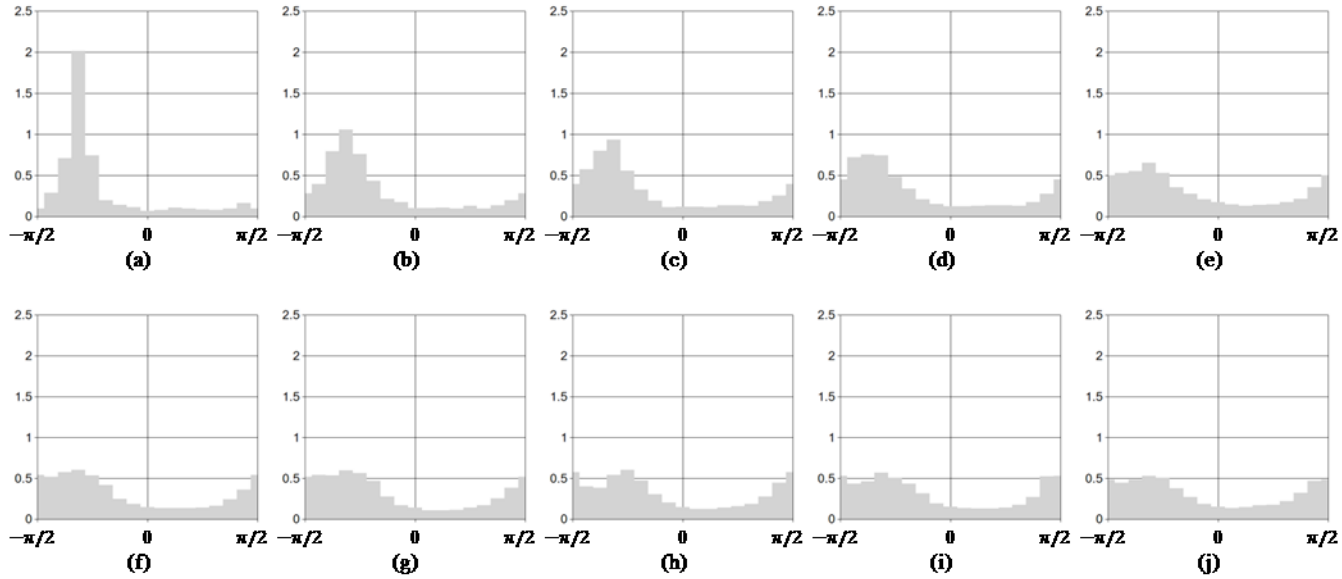

Figure 4. The histograms of the orientation angles at the purple point E in Fig. 1. The histograms are generated from data sets belonging to the ring-like region with distance of (a) 1–20, (b) 21–40, (c) 41–60, (d) 61–80, (e) 81–100, (f) 101–120, (g) 121–140, (h) 141–160, (i) 161–180, and (j) 181–200 pixels, respectively.

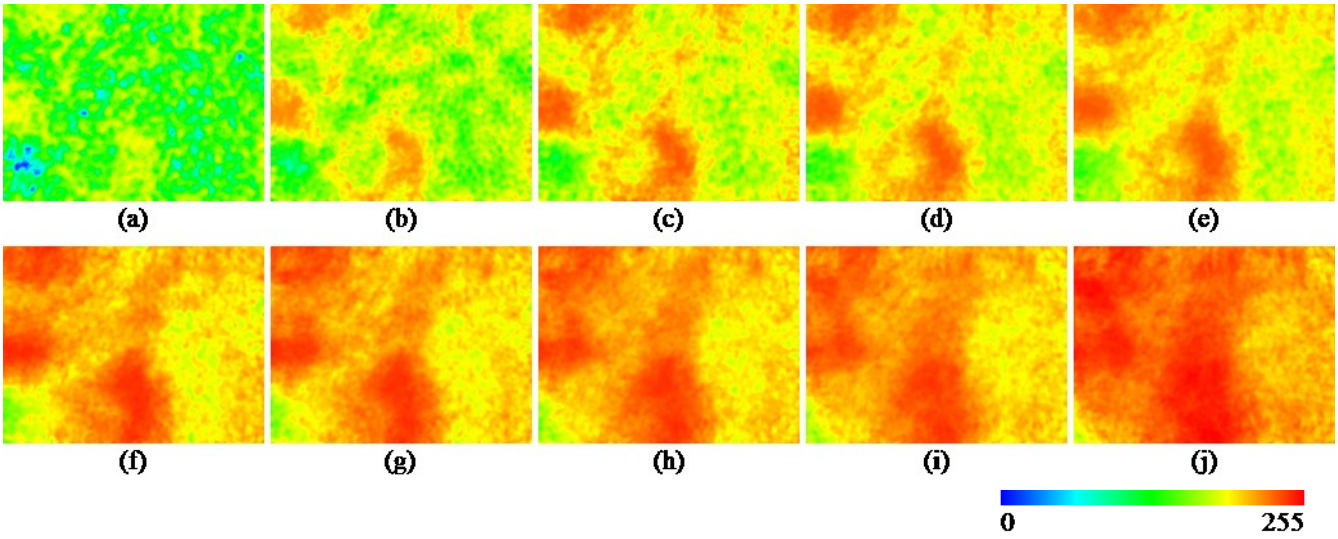

Figure 5. Entropy maps, which are calculated from the histogram for the regions with distance of (a) 1–20, (b) 21–40, (c) 41–60, (d) 61–80, (e) 81–100, (f) 101–120, (g) 121–140, (h) 141–160, (i) 161–180, and (j) 181–200 pixels. These are normalized from 1.61 to 3.89 in value in 256 pseudo color (refer to the colar bar at the bottom).

First suppose ten concentric circles with a center corresponding with the pixel of interest, and radii ranging from 0 to 200 pixels by every 20 pixels. We then prepare the eleven rings surrounded by the adjacent concentric circles, where the inner circle is not included, but the outer one is included in the region. The Euclidian distance from the pixel of interest to inner and outer circumferences are 1-20, 21-40, 41-60, 61-80, 81-100, 101-120, 121-140, 141-160, 161-180, and 181-200 pixels. The thickness of the ring was decided as 20 pixels so that the number of pixels belonging to each region amounts to more than 1000 pixels. Next, we generate the histograms of the direction in the individual ring regions. Here, the numbers of bins of the histograms are decided according to Sturges' formula [3]. Also, the histograms are normalized such that the total sum is a unity. The ten histograms are accompanied with the pixel of interest, which involve information on orientation at the pixel. Fig. 4 shows the histograms of orientation angles at point E shown in Fig. 1, where the vertical and horizontal axes are frequency and orientation angle, respectively. We observe that histogram deviation decreases with the increase of the distance from the center.

We introduce entropy to measure deviation of histogram. If the entropy is low, the histogram is deviated, that is, a high degree of orientation takes place. Conversely, the histogram approaches to a uniform distribution with the increase of the entropy, which represents a low degree of orientation. Then, entropy measures the degree of orientation. Fig. 5 shows the

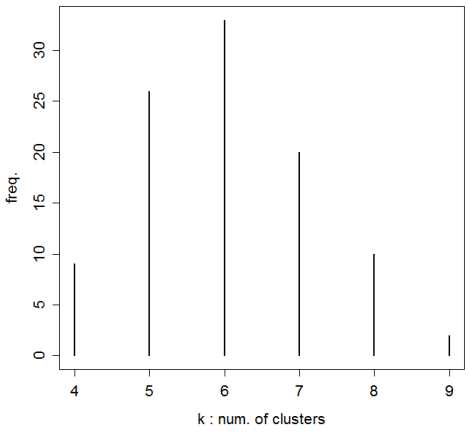

Figure 6. Gap statistics for the entropy. The horizontal and the vertical lines are number of clusters and frequencies.

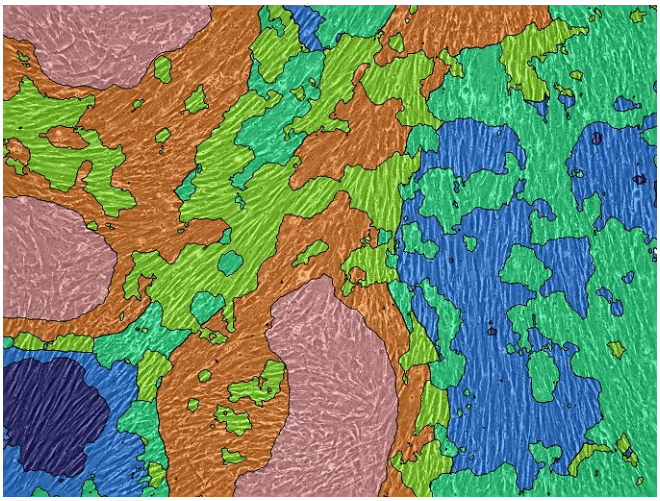

Figure 7. Segmentation result by the proposed algorithm

entropy maps in which pixel values are normalized throughout the whole images and represented in pseudo color of 256 scales. The maximum and minimum pixel values are 1.61 and 3.89, respectively. Figs. 5 (a), (b), (c), (d), (e), (f), (g), (h), (i), and (j) are entropy maps in ring regions, whose inner and outer radii are 1-20, 21-40, 41-60, 61-80, 81-100, 101-120, 121-140, 141-160, 161-180, and 181-200 pixels, respectively.

From Figs. 5 (a) to (j), the images turn more reddish and more homogeneous. This means that the degree of orientation decreases with an increase of the distance from a pixel of interest, because reddish color represents high entropy value. On the other hand, it is noteworthy that the color in the regions corresponding to regions A in Fig. 1 is very reddish in Figs. 5 (b) to (j). This means that the orientation is weak in those regions. Next, the color in the region corresponding to region B in Fig. 1 is relatively less reddish throughout Figs. 5. This means that the orientation is relatively high. Finally, although the color in the region corresponding to region C in Fig. 1 is reddish in Figs. 5 (c) to (j), the color of region C is relatively less reddish than that of regions A. This means that the entropy in region C varies more gradually than that in regions A.

C. *Segmentation*

We demonstrated that entropy is effective in measurement of the degree of orientation. However, it is inconvenient for biologists to intuitively understand how the cells are oriented, because each pixel has ten data according to the distance from the pixel. Segmentation based on the orientation will be effective in biologists' intuitive understandings. For this purpose, we first regard the ten data at each pixel as a ten-dimensional vector, and then employ *K*-means method for segmentation [4]. We decided the number of clusters using gap statistic [5]. Fig. 6 shows the results of gap statistic. From the figure, the number of clusters should be 6.

III. Result and Evaluation

We applied the proposed segmentation algorithm to the image in Fig. 1. Fig. 7 shows the segmented result. In Fig. 1, regions A correspond to regions where the orientations seem chaotic; Region B corresponds to region where the clear orientations are observed; Region C corresponds to a region where the two orientations seem to join. The proposed algorithm is desirable to at least differentiate these differences. We should note that the regions corresponding to regions A in Fig. 1 are segmented to regions which are represented in pink in Fig. 7. Also, the region corresponding to region B in Fig. 1 segmented to regions which are represented mainly in blue and green in Fig. 7. In addition, the regions corresponding to region C in Fig. 1 segmented to regions which are represented mainly in orange and light green in Fig. 7. It can be concluded from the result that the proposed algorithm differentiates these regions.

IV. Conclusion

This research considered a method to analyze the orientations of cell image at a confluent state, which has been seldom or never took up so far in the cell image analysis using computer having over 50 year history. Near-future evolution of iPS cell to regenerate medicine will cause the computer assisted cell image analysis to play a more important role than ever. Our futuristic work will be to investigate the effectiveness in detail by applying the method to variety kinds of confluent cell images.

References

[1] Y. Sato, S. Nakajima, N. Shiraga, H. Atsumi, S. Yoshida, T. Koller, G. Gerig, and R. Kikinis, "Three-dimensional multi-scale line filter for segmentation and visualization of curvilinear structures in medical images," *Medical Image Analysis*, vol. 2, no. 2, pp. 143-168, 1998.

[2] A. F. Frangi, W. J. Niessen, K. L. Vincken, M. A. Viergever, "Multiscale vessel enhancement filtering," *MICCAI*, Cambridge, MA, pp. 130-137, 1998.

[3] H. A. Sturges, "The choice of a class interval," *J. American Statistical Association*, pp. 65-66, 1926.

[4] C. M. Bishop, *Pattern Recognition and Machine Learning*, Springer-Verlag, 2006.

[5] R. Tibshirani, G. Walther, and T. Hastie, "Estimating the number of clusters in a dataset via the gap statistic," *Journal of the Royal Statistical Society B*, vol. 63, no. 2, pp. 411-423, 2001.