# A Segmentation Method for Counting Microbial Cells in Microscopic Image

Hak-Kyeong Kim, Sun-Hee Lee, Myung-Suk Lee and Sang-Bong Kim

Abstract: In this paper, a counting algorithm hybridized with an adaptive automatic thresholding method based on Otsu's method and the algorithm that elongates markers obtained by the well-known watershed algorithm is proposed to enhance the exactness of the microcell counting in microscopic images. The proposed counting algorithm can be stated as follows. The transformed full image captured by CCD camera set up at microscope is divided into cropped images of  $m \times n$  blocks with an appropriate size. The thresholding value of the cropped image is obtained by Otsu's method and the image is transformed into binary image. The microbial cell images below prespecified pixels are regarded as noise and are removed in the binary image. The smoothing procedure is done by the area opening and the morphological filter. Watershed algorithm and the elongating marker algorithm are applied. By repeating the above stated procedure for  $m \times n$  blocks, the  $m \times n$  segmented images are obtained. A superposed image with the size of  $640 \times 480$  pixels as same as original image is obtained from the  $m \times n$  segmented block images. By labeling the superposed image, the counting result on the image of microbial cells is achieved. To prove the effectiveness of the proposed method in counting the microbial cell on the image, we used *Acinetobacter sp.*, a kind of ammonia-oxidizing bacteria, and compared the proposed method with the global Otsu's method, the traditional watershed algorithm based on global thresholding value and human visual method. The result counted by the proposed method shows more approximated result to the human visual counting method than the result counted by any other method.

Keywords: adaptive automatic thresholding method, otsu's method, watershed algorithm, acinetobacter sp

# I. Introduction

In this paper, a counting algorithm hybridized with an adaptive automatic thresholding method based on Otsu's method and the algorithm that elongates markers obtained by the wellknown watershed algorithm is proposed to enhance the exactness of the microcell counting in microscopic images. The proposed counting algorithm can be stated as follows. The transformed full image captured by CCD camera set up at microscope is divided into cropped images of  $m \times n$  blocks with an appropriate size. The thresholding value of the cropped image is obtained by Otsu's method and the image is transformed into binary image. The microbial cell images below prespecified pixels are regarded as noise and are removed in the binary image. The smoothing procedure is done by the area opening and the morphological filter. Watershed algorithm and the elongating marker algorithm are applied. By repeating the above stated procedure for  $m \times n$  blocks, the  $m \times n$  segmented images are obtained. A superposed image with the size of 640×480 pixels as same as original image is obtained from the  $m \times n$  segmented block images. By labeling the superposed image, the counting result on the image of microbial cells is achieved. To prove the effectiveness of the proposed method in counting the microbial cell on the image, we used Acinetobacter sp., a kind of ammonia-oxidizing bacteria, and compared the proposed method with the global Otsu's method, the traditional watershed algorithm based on global thresholding value and human visual method. The result counted by the proposed method shows more approximated result to the human visual counting method than the result counted by any other method.

# II. Counting theory and the proposed algorithm 2.1 Problem statement

In this section, to enhance the exactness to count microbial cells on the image, the proposed method hybridized with adaptive automatic thresholding method based on optimal thresholding method by Otsu and watershed algorithm is described.

Because Syto13 not only becomes faded very fast within 2 ~3 seconds and the dye degree of each microbial cell is different but also background image has different gradient as shown in Fig. 1(a), it is difficult to apply the uniform thresholding value over the entire image. Fig. 1(b) is an image cropped from a rectangular part shown in Fig. 1(a). Furthermore, because binary image has a lot of overlapped image like Fig. 1(b), the microbial cell counting methods by general segmentation method have limit to count the microbial cells exactly.

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(a) Gray image,

(b) Overlapped image.

Fig. 1. Gray image and overlapped image.

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Fig. 2 shows the gray value distribution for the gray image of Fig. 1(a). Fig. 3 expresses the threshold values obtained through Otsu's method for full image of Fig. 1(a) and for block by block when the full image of Fig. 1(a) is separated as 2 × 2 blocks. As we can see from Fig. 4, we can see that the thresholding values are different according to the block positions of the image. Therefore, to get more precise counting result for microbial cells, it is more desirable to obtain the thresholding value for the separated blocks rather than for the global image. So, the purpose of this paper is to develop an algorithm that can count microbial cells exactly in spite of the overlapped shape, the variation of thresholding value and various gradients of microscopic image according to the image positions.

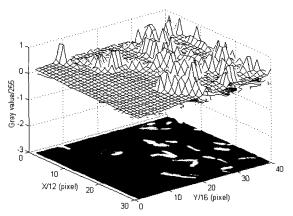


Fig. 2. The gray value distribution of Fig. 1(a)

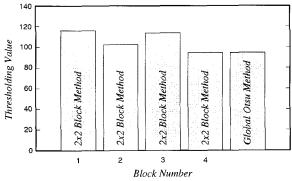


Fig. 3. Threshold values of the blocks.

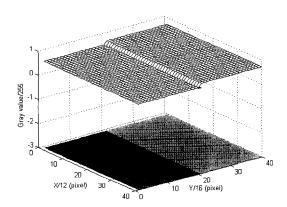


Fig. 4. Three-dimensional thresholding value of each block.

# 2.2 Ostu's thresholding method

In this section, we will show the Otsu's thresholding method that is usually applied to bimodal type histogram[3],[5],[8]. As shown in Fig. 3 and Fig. 4, the thresholding values are varied according to the positions in the captured image even though the microbial cell type is the same. So, we divide the full image into an appropriate block size with the same pixels and get the thresholding values for each block based on Otsu's method. To adopt the bi-thresholding method, the histogram of an image is converted to a probability distribution.

Let  $n_i$  denote the number of pixels at level i. Now if the total number of pixels is denoted by N, then,

$$N = \sum_{i=1}^{L} n_i \tag{1}$$

To facilitate the formulation, the gray-level histogram is normalized and a probability distribution of gray-levels is obtained:

$$p_i = \frac{n_i}{N}, \ p_i \ge 0, \ \sum_{i=1}^{L} p_i = 1$$
 (2)

Now, let the pixels be thought of as divided into two classes  $C_0$  and  $C_1$  (background and object or vice versa) which denote respectively the pixels with levels [1, 2, ---, t] and [t+1, ---, L], and t is the required threshold value. The probabilities of occurrence of classes  $C_0$  and  $C_1$  are given as respectively:

$$\omega_0 = \sum_{i=1}^t p_i = \omega(t) \tag{3}$$

$$\omega_1 = \sum_{i=t+1}^{L} p_i = 1 - \omega(t) = 1 - \omega_0 \tag{4}$$

Similarly, the means of classes  $C_0$  and  $C_1$  are calculated as respectively:

$$\mu_0 = \sum_{i=1}^t i p(i/C_0) = \sum_{i=1}^t \frac{i p_i}{\omega_0} = \frac{\mu(t)}{\omega(t)}$$
 (5)

$$\mu_1 = \sum_{i=t+1}^{L} ip(i/C_1) = \sum_{i=t+1}^{L} \frac{ip_i}{\omega_1} = \frac{\mu_T - \mu(t)}{1 - \omega(t)}$$
 (6)

where

$$p(i/C_0) = \frac{p_i}{\omega_0}$$
 ,  $p(i/C_1) = \frac{p_i}{\omega_1}$  (7)

which are the probabilities that gray level i come from the classes  $C_0$  and  $C_1$ , respectively. Total means in classes  $C_1$  are calculated as the following:

$$\mu_T = \sum_{i=1}^{L} i p_i \tag{8}$$

The zero and first order cumulative moments of the histogram up to the threshold level t are obtained as:

$$\mu(t) = \sum_{i=1}^{t} i p_i \tag{9}$$

The between-class variance for the classes and is calculated as follows:

$$\sigma_B^2 = \sigma^2(t) = \omega_0(\mu_0 - \mu(t))^2 + \omega_1(\mu_1 - \mu(t))^2$$

$$= \omega_0 \omega_1(\mu_0 - \mu_1)^2 = \frac{[\omega(t)\mu_T - \mu(t)]^2}{\omega(t)[1 - \omega(t)]}$$
(10)

The optimal threshold value t is calculated by maximizing the between-class variance for the classes  $C_0$  and  $C_1$ .

Total variation( $\sigma_T^2$ ) in class is as the following equation:

$$\sigma_T^2 = \sum_{i=1}^{L} (i - \mu_T)^2 p_i = \omega_0 \sigma_0^2 + \omega_1 \sigma_1^2$$
 (11)

The optimal thresholding value is the gray level when the following equation is maximized.

$$\eta = \frac{\sigma_B^2}{\sigma_T^2} \tag{12}$$

#### 2.3 Watershed algorithm

This section presents the standard definition of the water-shed[1],[6-7],[11]. For the purpose of segmentation, we then look for the crest lines of the gradient image. A way to characterize these lines is to apply the watershed algorithm to the modulus of the gradient image. We present here the classical algorithm for computing the watershed, in the case of a function defined in  $R^2$  on a digital grid, with discrete range of step functions[1],[6-7],[11]. A gray-tone image can be represented by a mapping function  $f: Z^2 \mapsto Z$ . Then f(x) is the gray value of the image at point x. The points of the space  $Z^2$  may be the vertices of a square or of a hexagonal grid. It is assumed that f is continuously differentiable. A section of f at level i is a set  $X_i(f)$  and  $Z_i(f)$  defined as:

$$X_i(f) = \{ X \in \mathbb{Z}^2 : f(x) \ge i \}$$
 (13)

$$Z_i(f) = \{ X \in \mathbb{Z}^2 : f(x) \le i \}$$
 (14)

The distance function is the shortest distance between pixel y and  $Y^c$  defined by [1]

$$\forall y \in Y, \quad d(y) = dist(y, Y^c)$$
 (15)

where Y is a set of markers and is its complementary set  $Y^c$ . Euclidian distance is taken at this time.

A section of d at level i is given by

$$X_i(d) = \{y : d(y) \ge i\} = Y \ominus B_i \tag{16}$$

where  $B_i$  is a disk of radius, and  $\Theta$  means erosion of Y.

We can calculate the geodesic distance  $d_X(x, y)$  between x and y as the length of the shortest path(if any) included in X and linking x and y as shown in Fig. 5(a) [1].

$$\begin{cases} d_X(x,y) = shortestlength(C_{xy}) & \text{if } x,y \in X \\ d_X(x,z) = \infty & \text{if } x \in X \text{ and } z \notin X \end{cases}$$
 (17)

where  $C_{xy}$  is all paths linking x and y in X.

Then, we calculate that the set of all points of X that are at a finite geodesic distance from Y

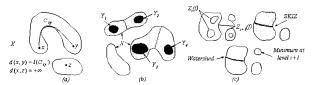


Fig. 5. (a) Shortest path and geodesic distance (b) SKIZ of a set Y in X (c) Watershed construction using geodesic SKIZ.

$$R_X(Y) = \{x \in X; y \in Y, d_X(x,y) : finite \}$$
 (18)

Next, we obtain SKIZ (geodesic skeleton influence zone) of Y in X that is the boundaries between the various influence zones as shown in Fig. 5(b) and (c).

$$Z_X(Y) = \{x \in X : d_X(x, Y_i) : finite$$

$$and \ \forall j \neq i, d_X(x, Y_i) < d_X(x, Y_i) \ \}$$

$$(19)$$

$$IZ_X(Y) = \bigcup Z_X(Y_i) \tag{20}$$

$$SKIZ_X(Y) = X / IZ_X(Y)$$
 (21)

where U and / stands for the set difference and union.

Finally, we build the watershed shown in Fig. 5(c) as follows: the minima of the function at height i+1,  $M_{i+1}(f)$  and the section at level for the catchment basins of f,  $W_i(f)$  are respectively given by:

$$M_{i+1}(f) = Z_{i+1}(f) / R_{Z_{i+1}(f)}(Z_i(f))$$
 (22)

$$W_{i+1}(f) = [IZ_{Z_{i+1}(f)}(X_i(f))] \cup M_{i+1}(f)$$
 where  $W_{-1}(f) = 0$ . (23)

At the end of the iteration process, the watershed line WL(f) is equal to:

$$WL(f) = W_N^c(f)$$
 (with max $(f) = N$ ) (24)

## 2.4 Proposed microbial cell counting algorithm

Fig. 6 describes the flow chart of the proposed algorithm where m and n are the number of the divided blocks for x and y coordinates.

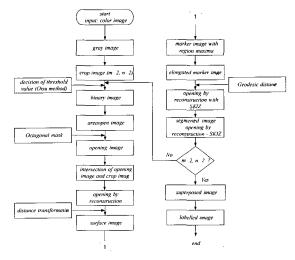


Fig. 6. The flow chart of the proposed algorithm.

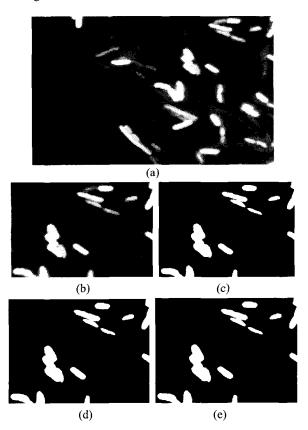
function 
$$h = elongated \_marker(f, l, \theta)$$
  
if  $|tan\theta| \le 1$   
 $s = sign(cos\theta)$   
 $x_1 = 0 : s : round(l cos\theta) - s$   
 $x_2 = round(x_1 tan\theta)$   
else  
 $s = sign(sin\theta)$   
 $x_2 = 0 : s : round(l sin\theta) - s$   
 $x_1 = round(x_2 / tan\theta)$   
 $X_1 = max(|x_1|), X_2 = max(|x_2|)$   
 $L_1 = 2X_1 + 1, L_2 = 2X_2 + 1$   
 $B = full(sparse(x_2 + X_2 + 1, x_1 + X_1 + 1, L_2, L_1))$   
 $h = open(open(f, B))$ 

where round, full, sparse and max are the functions of Matlab tool. l,  $\theta$ , f and h are the length, angle of marker and marker image and elongated marker image.

Function open(f, B) is the function opening f by B. If there is no B, B is given as follows:

$$B = \begin{bmatrix} 0 & 1 & 0 \\ 1 & 1 & 1 \\ 0 & 1 & 0 \end{bmatrix}$$

Fig. 7(b)  $\sim$  Fig. 7(l) show the results realizing the proposed algorithm for the right hand part of top after dividing Fig. 7(a) into  $2 \times 2$  blocks(m=2, n=2). Fig. 7(m) and Fig. 7(n) show the results combining the cropped images into one image and labeling the microbial cells.



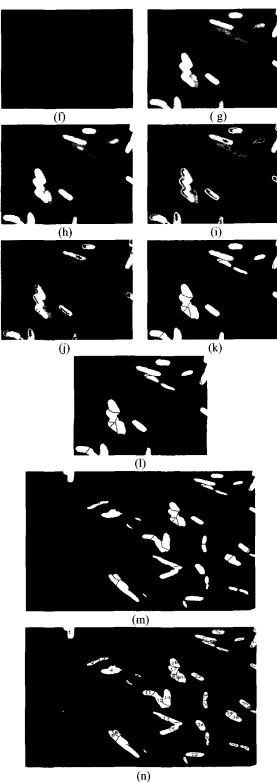


Fig. 7. The example applied to the proposed algorithm.

(a) Gray image (b) Cropped gray image (c) Binary image (d) Areaopen image (e) Opening image (f) Opening image on the crop image (g) Intersection image between opening image and gray image (h) Opening image by reconstruction (i) Marker image with region maxima (j) Marker image on elongated structures (k) SKIZ image with distance transformation and watershed algorithm(l) Segmented image (m) Superposed image (n) Labeled image

Fig. 7 describes the results of reconstructing the cropped images and labeling the microbial cells. The proposed algorithm is processed as the follows. The image signals captured by CCD camera set up at microscope are changed to the corresponding digital image signals by image grabber interface card and those are saved in the frame memory. Then the saved image is color image with 640 × 480 pixels and it is transformed into gray image as shown in Fig. 7(a). The transformed full image is divided into cropped images of  $m \times n$  blocks(Fig. 7(b)). The thresholding value of the cropped image is obtained by Otsu's method and the image is transformed into binary image(Fig. 7(c)). The microbial cell images below appropriately prespecified pixels are regarded as noise and removed in the binary image. The smoothing procedure is done by the area opening(Fig. 7(d)) and the morphological filter(Fig. 8(e)) where the mask is a type of octagonal as the following:

After catching the intersection between the opened image and the cropped gray image, the combined image as possible as similar to the original image is obtained(Fig.  $7(f) \sim Fig$ . 7(h)). The watershed algorithm is applied for the cropped block images and each microbial cell is segmented. That is, from the reconstructed image, we get the distance function of Eq. (15) and obtain the surface image and the region maximum points based on the function that are used as markers(Fig. 7(i)). Because markers produce over-segmentation, these markers are elongated in the long direction of object(Fig. 7(j)) by the previous stated algorithm. The SKIZ is got from the equation of geodesic distance  $d_X(x,y)$ , Eq. (17) based on this elongated marker images and the images with watershed lines (Fig. 7(k)) are produced from Eq. (24). And then, segmented images(Fig. 7(1)) are obtained by subtracting watershed lines from these images(Fig. 7(k)). We repeat the above stated procedure for  $m \times n$  blocks, and the  $m \times n$  segmented images are obtained(Fig. 7(1)). A superposed image (Fig. 7(m)) with the size of 640×480 pixels as same as original image is obtained from the  $m \times n$  segmented block images got by repeating the previous procedure. By labeling the superposed image, the counting result on the image of microbial cells is achieved(Fig. 7(n)).

#### III. The experimental result and discussions

# 3.1 Experimental condition of medium

In this paper, *Acinetobacter sp.*, a kind of ammonia-oxidizing bacterias[4] is used for experiment. First, we input  $1.5 \,\ell$  Bacto Nutrient Broth(Difco) in  $2.5 \,\ell$  bioreactor and sterilize them for 5 minutes at the condition of  $121 \,^{\circ}$ C in temperature, 151bs/in2 in pressure. And then, we inoculate 103CFU/m $\ell$  of *Acinetobacter sp.* in bioreactor, cultivate them at the temperature of  $30 \,^{\circ}$ C and agitate with velocity 7 rpm for air supply 0.4vvm, DO 0.1 and pH 6.8. We input Syto 13 into

1 m $\ell$  medium, mix it well and put it on the ambient air for 20 minutes. After that, by filtering through nuclepore PC membrane( $\phi$  1.3 cm, Costar, USA) by vacuum pump, we fix the nuclepore on slide glass and observe the microscopic image. And then, we get the microscopic images by CCD camera and frame grabber card. By human visual method, we count the microbial cells on the microscopic image after blocking the image at the constant size. Finally, we store the image and compare this visual method with global Otsu's method, traditional watershed algorithm based on global thresholding value, and the proposed method.

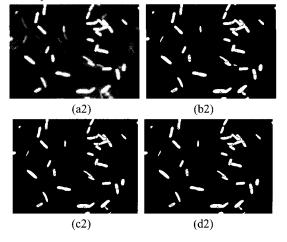
#### 3.2 Experimental results

In this paper, the experiment is done for the microbial cell counting algorithms such as global Otsu's method, traditional watershed algorithm based on global thresholding value, and the proposed method.

Fig. 8 shows the results that apply each algorithm. (a) shows gray image transformed from color image obtained from CCD camera, (b) shows image by global Otsu's method and (c) shows image by the global thresholding based on watershed algorithm and (d) shows image by the proposed algorithm.

(a1) (b1)

Sample 2: 10 times dilution after 9 hour cultivation



Sample 3: 100 times dilution after 21 hour cultivation

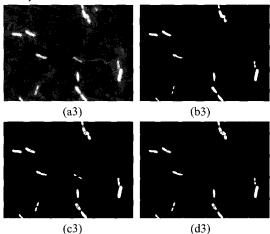


Fig. 8. Result images:(a) Original images, (b) Images by the conventional Otsu's method, (c) Images by the global thresholding based on watershed algorithm, (d) Images by the proposed method.

Fig. 9 shows the result of labeling the images (b), (c) and (d) in Fig 8. As shown in Fig.9, we can see that the number of the microbial cells counted by the proposed method shows the most approximated result to the cells counted by human visual method.

Of course, it is difficult to prove the effectiveness of the proposed method because the counting result by human visual method produces the errors depending on the counters although the cells counted by the proposed method is most approximate to the cells counted by human visual method. But because the proposed algorithm based on watershed algorithm proceeds to the segmentation similarly to counting by human visual method in spite of the overlapped image, the result is more reliable.

The denser the microbial cells are, the bigger the error between the proposed method and the other methods becomes. The density of the microbial cells and the gap of gray level between backgrounds according to the positions in an image must be small to count microbial cells exactly. To make lower the density of microbial cells, the increase of dilution rate and enough agitation must be desired. To decrease the gap of gray level on backgrounds, the development of excellent dying materials, exact dying methods, setting exact foci of lenses and removing noise are needed.

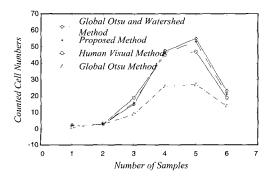


Fig. 9. Counting result of viable microbial cells.

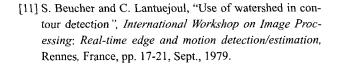
#### IV. Conclusion

In this paper, a counting algorithm combining an adaptive automatic thresholding method based on Otsu's method and the algorithm that elongates markers obtained by the wellknown watershed algorithm is proposed to enhance the exact microbial cell counting in microscopic images. The effectiveness of the proposed method in counting the microbial cell on the image is proved using Acinetobacter sp., a kind of ammonia-oxidizing bacteria, and the proposed method is compared to the global Otsu's method, the traditional watershed algorithm based on global thresholding value and human visual method. The result counted by the proposed method shows more approximated result to the human visual method than the result counted by any other method. The counting error is affected by microbial cell density and the gap of gray level in background. So to obtain more precise counting result, we need dying mycrocells exactly, the fast grasp of microscopic image, the enough agitation to have uniform distribution of microbial cells and removing background in getting images.

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