An Effective and Robust Method for Automatic Bacterial Colony Enumeration

Chengcui Zhang and Wei-Bang Chen
Department of Computer and Information Sciences
University of Alabama at Birmingham (UAB), Birmingham, AL 35226, USA
{zhang, wbc0522}@cis.uab.edu

Abstract

Bacterial colony enumeration has applications in many different assays such as antibiotic screening, toxicology testing, and genotoxicity measuring. The counting of bacterial colony is usually performed by well-trained technicians manually. However, this manual enumeration process has a very low throughput, and is time consuming and labor intensive in practice. To provide consistent and accurate results and improve the throughput, the existing colony counter devices and software were then developed and commercialized in the market. In this study, we propose a fully automatic colony counter and compare its performance with Clono-Counter, an existing automatic colony counter reported by Niyazi et al. Our proposed method can significantly reduce the manual labor by automatically detecting the dish/plate region and extracting and counting colonies. Our experimental results show that the proposed method outperforms Clono-Counter in terms of Precision, Recall, and F-measures.

1. Introduction

Bacterial colony enumeration has applications in many different assays such as antibiotic screening, toxicology testing, and genotoxicity measuring [1]. The main goal of study of these assays is to examine the survival rate of the microbes in a sample. The examination is achieved by pouring a liquefied sample containing microbes onto an agar plate, incubating the survived microbes as the seeds for growing the number of microbes to form colonies (a.k.a. colony forming unit - CFU) on the plate. The evaluation is done by counting the number of viable bacteria as colonies. These assays are broadly used not only in medical examinations, but also in the food and drug safety evaluations, environmental monitoring, and public health.

Bacterial colony counting process is usually performed by well-trained technicians manually. However, there might exist hundreds of colonies in a traditional 100mm petri dish as shown in Figure 1. Therefore, this manual enumeration process has a very low throughput and is time consuming and labor intensive in practice. In addition, the manual counting is an error-prone process since the counting results of the same plate obtained from different technicians might vary, especially when a vast number of colonies appear on the plate [2]. Another possible cause of variation is the judgment of the indistinguishable colony overlaps. Thus, it is important to have consistent criteria for measuring overlapped colonies.

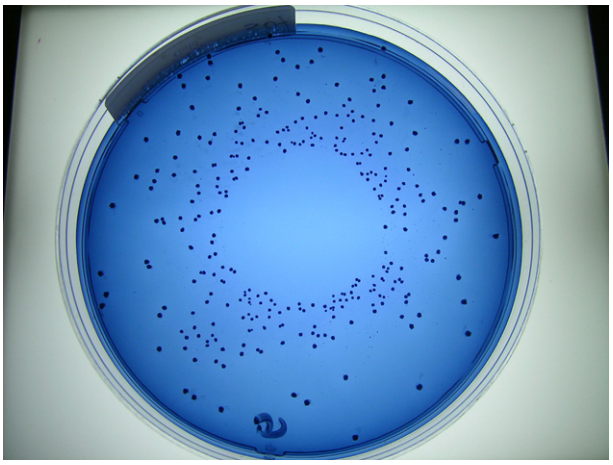

Figure 1. Bacteria colonies on a 100mm petri dish

To produce consistent and accurate results and improve the throughput, the existing colony counter devices were then developed and commercialized in the market [3]. In this study, we propose a fully automatic colony counter and compare its performance with Clono-Counter, an existing automatic counter reported by Niyazi *et al*. [4].

0-7695-2997-6/07 $25.00 © 2007 IEEE
DOI 10.1109/ICSC.2007.20

We reviewed the commercial colony counters available on the market and classified them into two categories.

The first kind of counter is often called automatic digital counters, and is widely used in most laboratories. However, they are not real automatic counters since they still require technicians use probe or pen to touch each colony so that the pressure sensor system can sense and register each count.

The second type of counter is semi-automatic or automatic counters. Typically, these counters are often very expensive. These high-priced devices often come with their own image capture hardware for acquiring high quality images to optimize counters' efficiency and performance. However, most laboratories cannot afford such expensive equipments since they perform assays only occasionally and must spend their limited budget on more cost-effective items and important projects. On the other hand, some laboratories that need to perform huge amount of enumeration tasks may require more than one high-throughput counter to fit their needs. Therefore, the colony enumeration device poses a significant budgetary challenge [5] to many laboratories.

In addition, some automatic colony counters accurately detect bacterial colony by adopting special growth media which contain fluorogenic substrates [8]. Growing bacteria metabolize the substrates, and then produce fluorescent product. These systems are extremely sensitive, and are good for detecting microcolonies. However, the fluorogenic substrates used in the media are costly, and the releasing fluorescent product can only be detected by using a sensitive instrument.

Besides, some automatic colony counters [4] still require users to manually specify the plate/dish area and provide parameters prior to the actual enumeration process. Some may need operators to adjust the threshold values in order to handle dishes/plates that differ from their default settings. In such cases, human operators are heavily involved in the operation, and it is thus not efficient for processing plates/dishes in a batch.

Further, laboratories always have needs to use different kinds of dishes and plates in their research. However, most of the commercial colony counters are designed for accommodating the most commonly used round shape petri dish with the diameter ranging from 60mm to 150mm. Therefore, these commercial products lack the flexibility for accommodating plates with different sizes and shapes.

Furthermore, some existing colony counters use only binary images for detecting colonies. Plenty of important characters of the bacterial colony, such as color, are lost for identifying the genus of the bacteria.

To overcome the above problems, our goal in this study is to design and implement an inexpensive, software-centered system which has the ability to process various types of plates and to correctly detect bacterial colonies on the plate. Also, this system can operate in a fully automatic manner without any human intervention. Thus, time and money can be saved for laboratories to do more important tasks.

Nowadays, digital cameras and flatbed image scanners become very popular and affordable in our daily life. These image acquiring devices provide a convenient yet inexpensive way to obtain high-quality images. For instance, a popular 5.0-megapixel digital camera can take photos with a resolution of 2592 × 1944. Moreover, some digital cameras embedded in mobile phones can have an image quality as high as 2.0-megapixel. Hence, the advances of digital imaging devices motive us to take advantages of these devices to obtain high-quality images for bacterial colony enumeration.

In this paper, we proposed an effective and robust system which can accept images obtained from these affordable devices for bacterial colony detection and enumeration in a fully automatic manner.

In the remaining of the paper, the proposed bacterial colony enumeration system architecture is introduced in Section 2. Section 3 demonstrates the experimental results. More discussions are presented in Section 4. Section 5 concludes this paper.

2. Methods

The proposed bacterial colony enumeration method consists of three core steps, including dish/plate region detection, colony isolation and separation, and colony enumeration. The first step automatically detects the dish/plate region in the image, and therefore, eliminates the need to manually select the target region by the operator. The second step isolates colonies on the dish/plate, and then, identifies clustered colonies and separates clustered colonies for the later enumeration step.

The main idea of the first two steps is to distinguish foreground objects (colonies) from the background. This process is also known as image segmentation whose goal is to minimize the intraclass variance of the foreground/background pixels, and to maximize the interclass variance. In our implementation, the image segmentation process is achieved by adopting Otsu's classification method [9] to find a proper threshold value for separating pixels into foreground and background classes in the target region, and then, determining the spatial connectivity of the foreground

pixels to retrieve individual colonies and separate clustered colonies.

In this section, we first introduce the details of the dish/plate region detection. Then, the colony isolation and separation process is described. Finally, the number of colonies in the image based on the objects detected in the segmentation results can be obtained.

2.1. Dish/Plate region detection

The motivation of developing a fully automatic dish/plate region detection function is to reduce the operator's workload by eliminating the manual process. Our goal is to enable the automatic detection of target regions (regions-of-interest) for various kinds of dishes and plates used in the experiments without manually selecting the target region in the image. The dish/plate detection can be a very difficult problem since the commonly used dishes/plates are transparent, and the shape and size of dish/plate may vary. Moreover, the contained culture medium may also vary in color.

Detecting the target area in a dish/plate with transparent medium is more difficult than those containing colored culture medium since the dish/plate region has almost the same color as the background. The empty dish/plate placed on a dark background has a more clear-cut look than placed on a bright background since the contrast is higher. Hence, we took pictures of various commonly used dishes/plates with transparent medium by placing them on a black surface such as counter top, fabric, or paper.

In our approach, we first apply the contrast-limited adaptive histogram equalization (CLAHE) on the converted grayscale images [6]. The CLAHE operates on small regions in the image rather than the entire image, thus making the dish/plate region conspicuous from the background.

In the next step, we would like to obtain a binary image that marks the dish/plate region as 1 (foreground) and background as 0 (background). To binarize image, we apply the Otsu's method [9] on the contrast adjusted image and obtained a threshold value. Those pixels with intensity values higher than the threshold will be labeled as 1 and the others as 0. Then we recognize those connected foreground pixels and identified them as the target region.

For some target regions detected this way, there may be small holes inside, and we fill in all the holes by adopting some morphology-based method and consolidate the target regions. Sometimes, this method can also detect some smaller objects that are not part of the target region. If the camera is reasonably calibrated and placed above the plate/dish, the target region should occupy the majority (and central) part of the image, thus we can use this assumption to remove those isolated small objects from the target region.

Figure 3 shows a few examples of plates/dishes that contain transparent medium and come with different sizes and/or shapes. The detected target regions of these plates/dishes, after applying the above steps, are also shown in Figure 3. It is worth noting that the transparent examples shown in this figure can be considered the 'worst cases' in detecting dish/plate regions for the purpose of colony enumeration. Detecting the target region in a dish/plate with colored medium is much easier because the color hue component evident in the target region can be used to separate the dish/plate region from the background.

2.2. Colony isolation and separation

The same ideas and steps mentioned in Section 2.1 can be applied to extract the medium surface and colonies from the target region. In this stage, we use the original color image (although some have transparent medium) for colony isolation and separation. Further, we adopt different algorithms to handle images with transparent and color media.

In order to distinguish images with transparent medium from colored ones, we examine the standard deviation of average RGB values from each color channel. The smaller the standard deviation is, meaning the variation of color is relatively low, the higher the possibility that the medium in the image is transparent.

2.2.1. Colony isolation. For images with colored medium, we calculate the color similarity in HSV (Hue-Saturation-Value) color space. The calculation of the color similarity is shown in Equation 1.

$$
\begin{aligned}
CS_{ij} &= 1 - \frac{1}{\sqrt{5}}\sqrt{(x_j - x_i)^2 + (y_j - y_i)^2 + (z_j - z_i)^2} \\
x_i &= S_i \times \cos(H_i \times 2\pi) \\
y_i &= S_i \times \sin(H_i \times 2\pi) \\
z_i &= V_i
\end{aligned}
\qquad (1)
$$

where CS_{ij} is the color similarity of two pixels i and j. H, S, V are the hue, saturation, and value of a pixel in the HSV color space. For images with transparent medium, we calculate color similarity only in its V (value) pane, since images with transparent medium lack variation in their hue (H) and saturation (S) components.

We calculate the color similarity values between a pixel and its eight neighbors and use the minimum value to detect the object boundaries.

After the calculation, the boundaries are more evident and the color similarity itself has formed a matrix as a grayscale image. Thus, we can adopt the Otsu's method used in the dish/plate region detection stage to obtain background and foreground objects as medium areas and colonies, respectively.

2.2.2. Clustered colonies separation. Ideally, an isolated foreground object from the previous step corresponds to one colony. However, such an object may correspond to more than one colony because several colonies may cluster together. Before we proceed with the colony enumeration stage, we have to split them apart in order to obtain the correct counting results.

To separate the connected colonies, we consider the intensities gradient image as a topological surfaces, thus we introduce the watershed algorithm to divide clustered colonies in the image as water flood in a topographical surface [7]. We illustrate the concept of watershed algorithm in Figure 2.

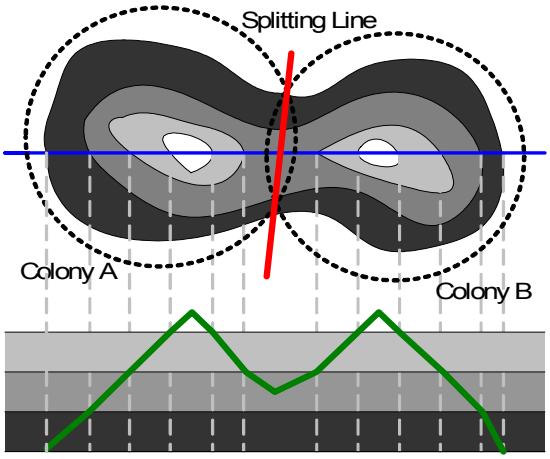

Figure 2. The concept of watershed algorithm

2.3. Colony enumeration

After all the colonies had been properly separated and identified, the last but not the least stage is to count the total number of viable colonies. This stage is relatively easy, and all we have to do is to add up the number of the objects that have been identified as colonies. However, the accuracy rate is crucial and the result can be useful only if all the previous stages have been well done.

3. Experimental results

To examine the device dependency in our experiments, we use three digital cameras as the image acquiring devices to obtain dish/plate images for bacterial colony detection. The three digital cameras include a Nikon D50 Digital SLR Camera (6.0-megapixel) with a resolution of 3008 × 2000, a Canon PowerShot A95 Camera (5.0-megapixel) with a resolution of 2592 × 1944, and a Sanyo DSC-J1 Camera (3.2-megapixel) with a resolution 1600 × 1200.

In addition, we use two different kinds of plates as our test cases. The first type of plate is obtained from the Department of Pediatric Dentistry at the University of Alabama at Birmingham. This type of plate contains the blue color Mitis-Salivarius agar which is used for isolating Mutans Streptococci. These acid-producing bacteria attack tooth enamel minerals and cause dental caries. The second type of plate is obtained from the Division of Nephrology, Department of Medicine, University of Alabama at Birmingham. This type of plate contains the transparent LB agar which is widely used in laboratories for *Escherichia Coli* culture.

3.1. Dish/Plate region detection

In our dish/plate region detection experiment, we collected six kinds of commonly used dishes and plates in the laboratory. We demonstrate these dishes and plates in Figure 3. In Figure 3, the first row shows the 140mm dish (A-1), 50mm dish (B-1) and 35mm dish (C-1), and the third row demonstrates the 96-well plate (D-1), 24-well plate (E-1) and 6-well plate (F-1).

We applied the proposed dish/plate region detection algorithm on these images for extracting the dish/plate regions, and the corresponding results are illustrated in the second and fourth rows in Figure 3.

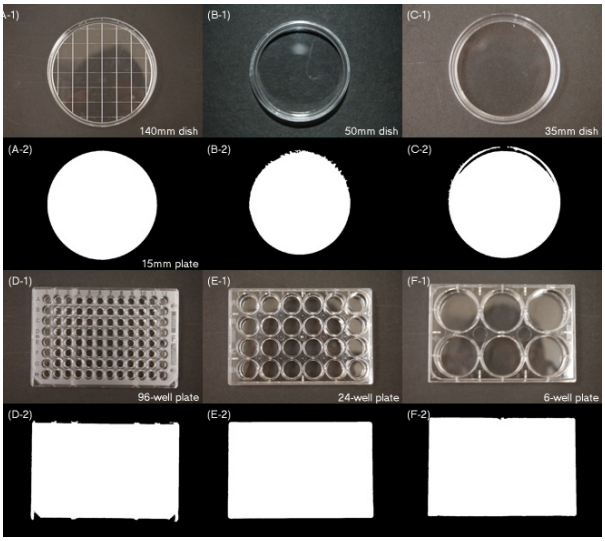

Figure 3. Dish/plate regions detected in images

The proposed automatic dish/plate region detection algorithm recognizes the background and foreground regions, marked as black and white, respectively. The experimental results show that our automatic dish/plate region detection algorithm is effective in detecting dish/plate regions regardless of the size and shape of the dish/plate. Thus, the proposed algorithm is flexible enough to be used for handling various kinds of dishes/plates.

3.2. Colony detection

In this experiment, we compare the proposed colony detection algorithm with both human counters and Clono-Counter which is a java program implemented and reported by Niyazi in 2007 [4].

In order to compare the ability to handle different kinds of media, we collected two sets of images, images with color and transparent medium, respectively. In addition, since Clono-Counter only accepts grayscale images at 200dpi, for a fair comparison, we converted the color images into grayscale images and resized the images in order to meet the requirements.

We test the same images with our proposed method and with Clono-Counter, and evaluate the performance of both methods in terms of precision, recall, and F-measure. The higher the value of these measures, the better the performance is. The details of the experiment and the experimental results are illustrated below.

First, we apply the proposed counter (P.C.) and Clono-Counter (C.C.) on three images with blue medium contained in the dish/plate. The experimental results are shown in Table 1. The proposed counter performs very well on the blue medium dish/plate. Its average precision, recall, and F-measure values are 0.97, 0.96, and 0.96, respectively. The performance of Clono-Counter is also shown in Table 1. Its average precision, recall, and F-measure values of it are 0.52, 0.99, and 0.67, respectively. The experimental results show that the proposed counter significantly outperforms the Clono-Counter.

Table 1. Performance on color medium

ID	Precision		Recall		F-measure	
	P.C.†	C.C. ‡	P.C. †	C.C. ‡	P.C. †	C.C. ‡
8358	0.98	0.62	0.95	0.98	0.97	0.76
8362	0.93	0.30	1.00	1.00	0.97	0.46
8389	0.99	0.66	0.93	0.99	0.96	0.79
Mean	**0.97**	**0.52**	**0.96**	**0.99**	**0.96**	**0.67**
Std	**±0.03**	**±0.19**	**±0.04**	**±0.01**	**±0.01**	**±0.18**

†. Proposed Counter
‡. Clono-Counter

We then apply the proposed counter (P.C.) and Clono-Counter (C.C.) on five images with transparent medium contained in the dish/plate. The experimental results are shown in Table 2. The proposed counter performs worse on the transparent medium than that of the blue medium. Its average precision, recall and F-measure values are 0.31, 0.97, and 0.45, respectively. The performance of the Clono-Counter is also shown in Table 2. Its average precision, recall and F-measure values are 0.00, 0.00, and 0.00, respectively. The zero values indicate that Clono-Counter failed to recognize any colony on the plate. There are two possible causes for this failure: 1) colonies in transparent medium are much more difficult to detect because they are almost indistinguishable from the background unless carefully checked by human eyes; and 2) transparent medium dishes/plates often come with a lot of noise such as bubbles and light reflection. However, the experimental results in Table 2 show that the proposed counter can handle, although not perfect, more difficult cases such as transparent medium dishes/plates.

Table 2. Performance on transparent medium

ID	Precision		Recall		F-measure	
	P.C.†	C.C. ‡	P.C. †	C.C. ‡	P.C. †	C.C. ‡
0029	0.11	0.00	1.00	0.00	0.20	0.00
0030	0.24	0.00	0.95	0.00	0.38	0.00
0031	0.40	0.00	0.95	0.00	0.56	0.00
0032	0.36	0.00	1.00	0.00	0.54	0.00
0033	0.43	0.00	0.95	0.00	0.59	0.00
Mean	**0.31**	**0.00**	**0.97**	**0.00**	**0.45**	**0.00**
Std	**±0.13**	**±0.00**	**±0.03**	**±0.00**	**±0.16**	**±0.00**

†. Proposed Counter
‡. Clono-Counter

3.3. Splitting clustered colonies

In the process of detecting colonies, there exist some clustered colonies that need to be further divided into single colonies. As mentioned earlier, we adopt the watershed algorithm to deal with this problem and found it useful in separating connected colonies in our experimental results. We give an example of the splitting performance of watershed algorithm in Figure 4.

In our experiment, we checked the performance of the watershed algorithm on 19 segments with clustered colonies which contain 98 colonies. After applying the watershed algorithm, we obtain 96 colonies. Only 2 overlapped colonies are missed in the splitting process. It is worth noting that the watershed algorithm is an integral part of the proposed system, in which each

step contributes to the better performance of the following steps.

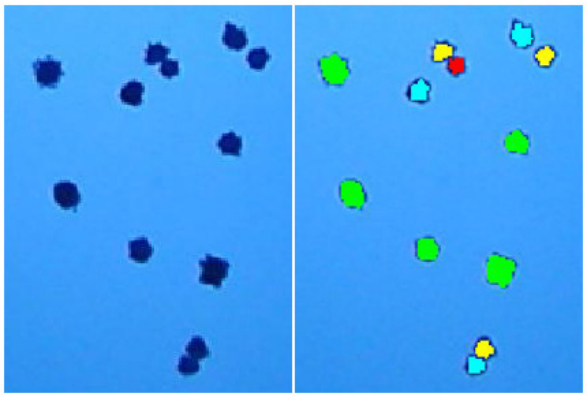

Figure 4. Clustered colonies split by Watershed algorithm

4. Conclusions and Discussions

The proposed automatic colony counter is a robust yet effective method for bacterial colony enumeration. It has the ability to detect the dish/plate regions, isolate colonies on the dish/plate, and further, separate the clustered colonies for accurate counting of colonies. Our proposed counter can handle various kinds of dish/plate, and is thus very flexible, making it attractive to laboratories. In addition, our counter operates automatically without any human intervention. The performance of the proposed counter is promising, especially when processing the dish/plate with color medium.

In our experiments, we found it much more difficult to deal with transparent medium since the growing colonies look very similar to the background. In addition, there exist a lot of noises on the plate such as bubbles, small scratches, and small markers. These round shape objects are very similar to the colonies and sometime it is hard to tell whether or not they are colonies even by human eyes. This makes the colony isolation task extremely difficult. Therefore, in our future work, we will put more effort on distinguishing the colony from noises for dishes/plates with transparent medium.

5. Future work

Though our method has outperformed the Clono-Counter, there are always rooms for further improvement. As mention in Section 4, the recognition of colony and noise can be further improved by introducing more colony features. We may use not only the size, shape, and color, but also some other features such as the transparency and topology of the colony. In addition, we plan to handle different species of bacterial colonies in a single plate. Our goal is to accurately classify different kinds of bacterial colonies and produce the correct count for each class.

6. References

[1] X. Liu, S. Wang, L. Sendi, and M. J. Caulfield, "High-throughput imaging of bacterial colonies grown on filter plates with application to serum bactericidal assays," *Journal of Immunological Methods*, vol. 292, pp. 187-193, 2004.

[2] C. W. Chang, Y. H. Hwang, S. A. Grinshpun, J. M. Macher, and K. Willeke, "Evaluation of Counting Error Due to Colony Masking in Bioaerosol Sampling," *Applied and Environmental Microbiology*, vol. 60, pp. 3732-3738, 1994.

[3] J. Dahle, M. Kakar, H. B. Steen, and O. Kaalhus, "Automated counting of mammalian cell colonies by means of a flat bed scanner and image processing," *Cytometry A*, vol. 60, pp. 182-188, 2004.

[4] M. Niyazi, I. Niyazi, and C. Belka, "Counting colonies of clonogenic assays by using densitometric software," *Radiation Oncology*, vol. 2, pp. 4, 2007.

[5] M. Putman, R. Burton, and M. H. Nahm, "Simplified method to automatically count bacterial colony forming unit," *J Immunol Methods*, vol. 302, pp. 99-102, 2005.

[6] Z. Karel, "Contrast limited adaptive histogram equalization," in *Graphics gems IV*: Academic Press Professional, Inc., 1994, pp. 474-485.

[7] V. Luc and S. Pierre, "Watersheds in Digital Spaces: An Efficient Algorithm Based on Immersion Simulations," *IEEE Trans. Pattern Anal. Mach. Intell.*, vol. 13, pp. 583-598, 1991.

[8] http://www.colifast.no/

[9] N. Otsu, "A Threshold Selection Method from Gray-Level Histograms," *IEEE Trans. on Systems, Man, and Cybernetics*, vol. 9, no. 1, pp. 62-66, 1979.