Automated Bacterial Colony Counting for Clonogenic Assay

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ABSTRACT

Bacterial colony enumeration is an essential tool for many widely used biomedical assays. This chapter introduces a cost-effective and fully automatic bacterial colony counter which accepts digital images as its input. The proposed counter can handle variously shaped dishes/plates, recognize chromatic and achromatic images, and process both color and clear medium. In particular, the counter can detect dish/plate regions, identify colonies, separate aggregated colonies, and finally report consistent and accurate counting result. The authors hope that understanding the complicated and labor-intensive nature of colony counting will assist researchers in a better understanding of the problems posed and the need to automate this process from a software point of view, without relying too much on specific hardware.

INTRODUCTION

Numerous dental diseases such as dental caries and periodontal diseases are closely related with the bacteria in our oral cavity. Taking dental caries as an example, dental caries is well-known a multi-factor disease, which occurred with both fermentable dietary carbohydrate and dental plaque bacteria. The *Mutans Streptococci*, one of the bacteria strains in our oral cavity, has been implicated as a major etiological agent of dental caries. Hence, it is critical to know what bacterial strains and the amount of them that are in the collected oral samples, such as the saliva and plaque samples. To identify and quantify the microbes in a sample, one of the most widely accepted function assay in both clinical and research laboratories, is the clonogenic assay (a.k.a. colony forming assay).

The clonogenic assay is achieved by pouring a liquefied sample containing microbes onto agar plates, incubating the survived microbes as the seeds for growing the number of microbes via binary fission to form colonies (colony forming unit, CFU) on the plates. The bacteria species can be distinguished by growing them on different selective medium, and the quantification for the amount of viable microbes in the sample can be measured by enumerating the number of colony on the plate, since each viable microbe can grow and become a colony. In this way, the identity and the quantity of the bacteria in a given sample can be determined. With this diagnostic tool, we can monitor the progress of the disease and even to indicate the susceptibility of future occurrence of the disease. Moreover, it also provides a basis to determine proper antibiotic agents used in medical treatment. Without a doubt, this assay is broadly used in biomedical examinations, food and drug safety test, environmental monitoring, and public health (Liu, Wang, Sendi, & Caulfield, 2004).

Though clonogenic assay is very useful, there exists a bottleneck that limits its throughput. The technical hurdle occurs at the final step, the colony enumeration step in the assay since it is a time consuming and labor intensive process. The reason is that there might exist hundreds or thousands of colonies in a Petri dish, but the counting process is manually performed by well-trained technicians. In

Figure **1** (A) and (B), we show a typical 100mm Petri dish with hundreds of colonies grown in it.

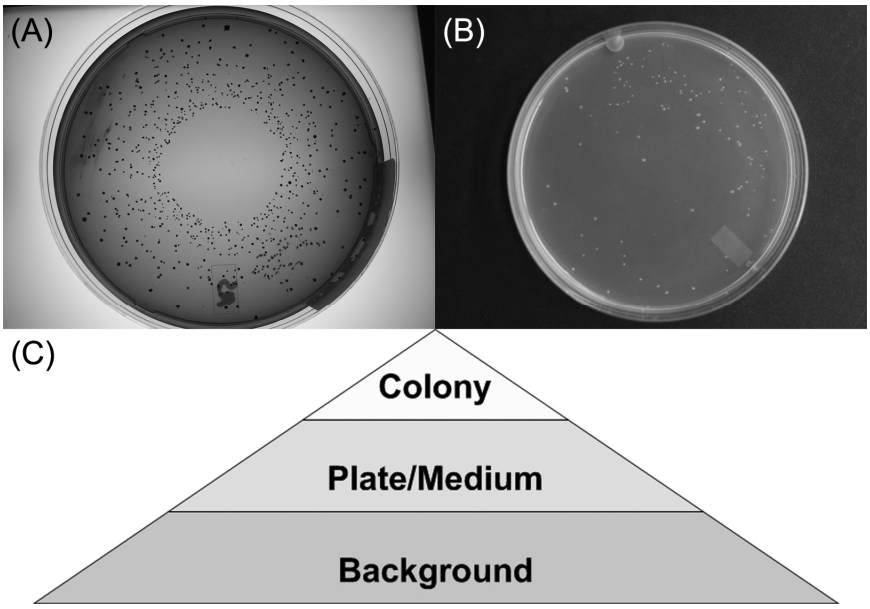


Figure 1. (A) *Mutans Streptococci* grown in a 100mm Petri dish with Mitis-Salivarius agar; (B) *Escherichia Coli* grown in a 100mm Petri dish with LB agar; (C) The hierarchical structure of objects in a bacterial colony image.

In addition to the low throughput problem, the consistency is another issue. The manual counting is an error-prone process since the results tend to have more subjective interpretation and mostly rely on persistent practice, especially when a vast number of colonies appear on the plate (Chang, Hwang, Grinshpun, Macher, & Willeke, 1994). Thus, it is very important to have consistent criteria to avoid the fluctuations in results.

In order to increase the throughput and to provide consistent and accurate results, colony counting devices were invented in the market (Dahle, Kakar, Steen, & Kaalhus, 2004). We reviewed these counters available on the market (Advanced Instruments, ; Barloworld Scientific ; BioLogics ; ChemoMetec ; Colifast ; Neutec Group ; Oxford Optronix ; Perceptive Instruments ; Progen Scientific ; Synbiosis) and classified them into two categories.

The first kind of counter, the automatic digital counters (Barloworld Scientific), is widely used in most laboratories. However, they are not truly automatic since they require operators to identify each colony with a probe so that the sensor system can detect and register each count. Obviously,

this kind of counter still heavily depends on the routine manual counting, and is of no use in solving the problems aforementioned.

The second type of counter is semi-automatic or automatic counters. Some counters of this type may require users to manually specify the plate/dish area and to provide parameters prior to the actual enumeration process (Niyazi, Niyazi, & Belka, 2007). Besides, some of them may need operators to manually adjust the threshold values in order to handle dishes/plates that differ from their default settings. In addition, most automatic counters can only process one Petri dish at a time. In such cases, human operators are still heavily involved in the operation, and it is thus not efficient for high-throughput processing of plates/dishes.

In addition to the high-throughput processing concern, the affordability is another issue. Most automatic counters are often very expensive since they are equipped with dedicated image capturing hardware for acquiring high quality images to optimize counters' efficiency and performance. Besides, some automatic counters can only accurately detect colonies by growing bacteria on special growth medium with fluorogenic substrates (Dee et al., 2000). The substrates are utilized by bacteria and are metabolized into fluorescent product that can be used to locate colonies. These systems are extremely sensitive, and are good for detecting micro-colonies. However, the disadvantages are that it is costly to add fluorogenic substrates in the medium, and a sensitive instrument is also required to detect the fluorescence signal. Hence, the affordability of this kind of equipment is still questionable for most of laboratories due to the economical inefficiency of these high cost equipments in the market. For some laboratories that need to perform a large amount of enumeration tasks, more than one high-throughput counter are needed to fit their needs. Thus, the colony enumeration device poses a significant budgetary challenge to many laboratories (Putman, Burton, & Nahm, 2005).

Further, the robustness and applicability of the existing automatic counters is another concern. Laboratories have needs to use various types of dishes and plates in their examinations. However, most of the commercial counters are designed for measuring 60-150mm Petri dish and thus lack the flexibility for accommodating plates with different sizes and shapes. In addition, some existing counters use only binary images for detecting colonies. Plenty of important characters of the colony, such as color, are lost for identifying the genus of the bacteria.

Nowadays, digital image capture devices such as digital cameras and flatbed scanners, become more popular and affordable. Hence, it motives us to use these devices to obtain high-quality images for counting bacterial colonies. In this chapter, we use photos taken by digital cameras with various settings as our input images, and then, make an attempt to recognize colonies in those images. Our goal is to provide an inexpensive, software-based solution for alleviating those problems aforementioned. The proposed colony enumeration system is designed and implemented to work in a fully automatic manner such that it can process various types of plates and correctly detect bacterial colonies without users' intervention. Thus, time and money can be saved for laboratories to do more important tasks.

In the rest of this chapter, we first introduce the system architecture and the proposed methods, then demonstrate the experimental results, and finally summarize and conclude this chapter.

METHODS

The proposed bacterial colony enumeration system simulates the human recognition behavior that progressively identifies objects in an image based on the hierarchical layout of major objects in a bacterial colony image. The natural hierarchy of objects in a bacterial colony image consists of three layers, including the background, the plate/medium region, and the colonies. We illustrate the hierarchical layout of a bacterial colony image in Figure 1 (C). More specifically, the bacterial colony enumeration system first separates the plate/medium region from the background, and then, recognizes the bacterial colonies in the identified plate/medium region.

There are four major steps in the proposed bacterial colony enumeration system: dish/plate region detection, colony recognition, clustered colony separation, and finally, the colony enumeration. The main technique used in the proposed system is object segmentation. In particular, the proposed segmentation technique distinguishes foreground objects, such as dish regions or colonies, from their corresponding background by minimizing the intraclass variance and maximizing the interclass variance. In this chapter, we introduces a progressive segmentation method which is based on the recursive use of a widely adopted clustering and thresholding method called Otsu's method (Otsu, 1979). This method is used to find a proper threshold value for separating pixels into foreground and background classes in the target region (regions-of-interest). Once all colonies on the plate are recognized, we can simply count the number of remaining segments in the image as the estimated number of bacterial colonies on the plate/dish. In the remaining of this section, we will introduce in details the proposed bacterial colony enumeration system.

Dish/Plate Region Detection

The first step in this system is to detect the dish/plate region in a given image. The goal is to reduce the operator's workload by eliminating the process of manually specifying the target dish/plate region in the image. To make the dish/plate region more conspicuous from the background, the contrast-limited adaptive histogram equalization (CLAHE) is first performed on the converted grayscale images which operates on small regions called tiles in the images rather than the entire image (Zuiderveld, 1994). The contrast of each tile is enhanced and the neighboring tiles are then combined using bilinear interpolation to eliminate artificially induced boundaries.

Following the histogram equalization, we apply the Otsu's segmentation algorithm on the contrast enhanced image to detect the dish/plate region as a target region. However, because of the varying intensity of the target object (dish/plate region), there may form some small holes inside the target plate region. Those small holes are then filled by adopting a morphology-based method, and the target region can thus be consolidated. Sometimes, this method may also detect some smaller objects outside the target region due to the existence of noise in the background region. These isolated small objects are also removed by our algorithm on the basis of the assumption that the target region should occupy the majority (and central) part of the image.

With the above operations, the target plate/dish regions can be correctly detected most of the time. We demonstrate the effectiveness of our automatic dish/plate region detecting algorithm in the experimental results (as shown in Figure 3). The experimental results show that our proposed counter can detect dish/plate regions very effectively, regardless of the size and shape of the dish/plate. Thus, the proposed system is flexible enough to be used for handling various kinds of dishes and plates.

Colony Recognition

The second step in our proposed architecture is colony recognition. The purpose of this step is to isolate colonies in the dish/plate, identify clustered colonies, and separate clustered colonies for subsequent colony enumeration.

Before actually performing the colony recognition, we have noticed that bacterial colony images can be classified into two groups based on their color characteristics. For those images with abundant color information, we call them chromatic images. On the contrary, for those images with less color information, we call them achromatic images.

Figure **1** (A) and (B) shows black *Mutans Streptococci* colonies grown in the 100mm Petri dish with the blue Mitis-Salivarius agar, and white *Escherichia Coli* colonies grown in the 100mm Petri dish with the clear LB agar, respectively. These two images exemplify the chromatic image and the achromatic image. Since these two types of images are quite dissimilar in their color characteristics, it is more appropriate to handle chromatic and achromatic images in different ways.

In order to distinguish chromatic images from the achromatic ones, we examine the standard deviation of average RGB values from each color channel. The smaller the standard deviation is, indicating a relatively low variation of colors, the higher the possibility that the image is achromatic. After grouping images into the two groups (chromatic/achromatic) by their embedded color information, we are now ready to apply different algorithms on extracted dish/plate regions to isolate colonies.

For chromatic images, we not only use Otsu's method (Otsu, 1979) to separate colonies from medium, but also adopt color similarity in HSV (Hue-Saturation-Value) color space to facilitate the colony boundary detection (Ma & Zhang, 1998). This is essential since a simple global threshold cannot extract all colonies due to the existence of artifacts such as scratches, dusts, markers, bubbles, reflections, and dents in the image. Equation 1 shows the calculation of color similarity in the HSV color space.

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

where CS_{ij} is the color similarity of two pixels i and j. H, S, and V are the hue, saturation, and value of a pixel in the HSV color space.

The use of color similarity in colony boundary detection is based on the assumption that pixels inside a segment, no matter it is a colony segment or a dish/plate segment, have higher similarity values with its neighboring pixels, and pixels along the segment boundary have lower similarity values with their neighbors. For each single pixel, it is surrounded by eight neighboring pixels such that all nine pixels form a 3×3 window. We calculate the color similarity values between a pixel and its eight neighbors, and use the minimum similarity value to represent the maximum color difference with its neighbors. Thus, pixels within a segment have higher minimum similarity values. On the contrary, pixels on the boundary of a segment have lower values. After the calculation, the boundaries/edges are more evident, and all the minimum color similarity values form an intensity image. Thus, we can adopt the Otsu's method on the extracted dish/plate region in the enhanced intensity image to further distinguish the background (medium) from foreground objects (candidate colonies).

A much more challenging part in this research is to deal with achromatic images. Most of the existing colony counters have disappointing performance in handling achromatic images due to the low contrast between colonies and medium. In addition, the background artifacts look very similar to colonies in the clear agar, making it more difficult to discriminate the background artifacts from real colonies in the dish/plate.

To handle achromatic images, we develop a different method to alleviate the low contrast and artifacts problems. We also apply Otsu's segmentation algorithm to isolate colonies. However, Otsu's method is much less accurate in achromatic images due to the interference of artifacts. An additional noise removal step is developed for those achromatic images.

The color similarity measurement described earlier in this section cannot be applied since achromatic images lack color information. In this chapter, we proposed a new statistic approach to detect and remove those artifacts and successfully preserve only colonies. Our proposed statistic approach includes two steps. The first step is to remove those relatively large artifacts. We collect the sizes of all objects detected by Otsu's method from the dish/plate region, and generate frequency distribution with log base of those size values. Colonies of similar size should occupy the high frequency segment in this distribution, and the frequencies for those very large artifacts should be very low. By this assumption, we can remove those large size objects. The next step is to remove those smaller artifacts which are about the same size as the colonies in the dish/plate. In this step, the area size is not a good determinant since the area size range of those small artifacts has a significant overlap with that of colonies. Instead, we consider the intensity distribution of the dish/plate region as a two-peak distribution which consists of the distribution of medium pixels (background) and distribution of colonies pixels (foreground). Those small artifacts belong to background distribution; however, they have overlapped with the colony distribution. Therefore, we assume that colonies should be significantly different in intensity values comparing with their surrounding background, and it is very likely that those small artifacts have similar intensity values to their surrounding pixels. Based on this assumption,

we examine each small object including colonies by hypothesis testing. In the hypothesis testing, we use the mean of surrounding pixel values as null and test if the mean of object pixel values has significant difference with the null, at the significance level of $\alpha = 0.01$.

Colony Separation

After most of the artifacts being removed from both chromatic and achromatic images, the remaining foreground objects are considered to be colonies. Ideally, each of these isolated foreground objects corresponds to one single colony. However, such an object may correspond to more than one colony because several colonies may cluster together. Therefore, there is a need to split them in order to obtain the correct colony count. To separate the aggregated colonies, we consider the intensity gradient image as topological surfaces (Vincent & Soille, 1991), thus the Watershed algorithm can be applied to divide clustered colonies in the image just as water flood in a topographical surface. We demonstrate how Watershed algorithm works in Figure 2. Figure 2 (A) shows a binary image that simulates two clustered colonies; Figure 2 (B) presents the distance transform of the binary image in Figure 2 (A); Figure 2 (C) illustrates the Watershed transform successful splitting the clustered colonies. After applying the Watershed algorithm, almost all colony segments have been separated and identified and are ready for the colony enumeration step.

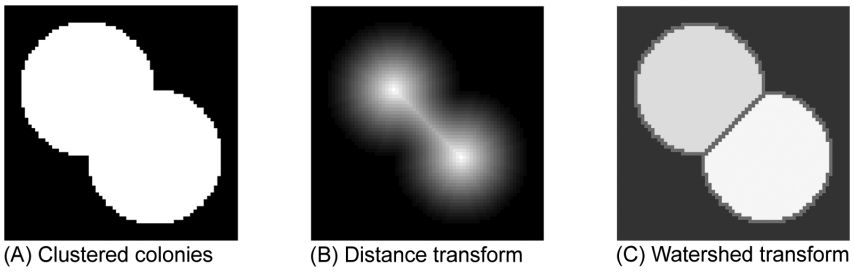


Figure 2. An example of the Watershed algorithm performed on clustered colonies

Colony Enumeration

After all colonies are properly separated and identified, the final step is to acquire the total number of viable colonies by adding up the number of the remaining objects that have been identified as colonies.

EXPERIMENTAL RESULTS

To test the robustness of this counting system, we use five different digital cameras as the image acquiring devices in our experiments to obtain bacterial colony images for bacterial colony enumeration. The five digital cameras include a Sony DSC T100 Digital Camera (8.0-megapixel) with a resolution of 3264 × 2448, 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, a Sanyo DSC-J1 Camera (3.2-megapixel) with a resolution 1600 × 1200, and an Asus P525 PDA cell phone built-in camera (2.0-megapixel) with a resolution 1600 × 1200.

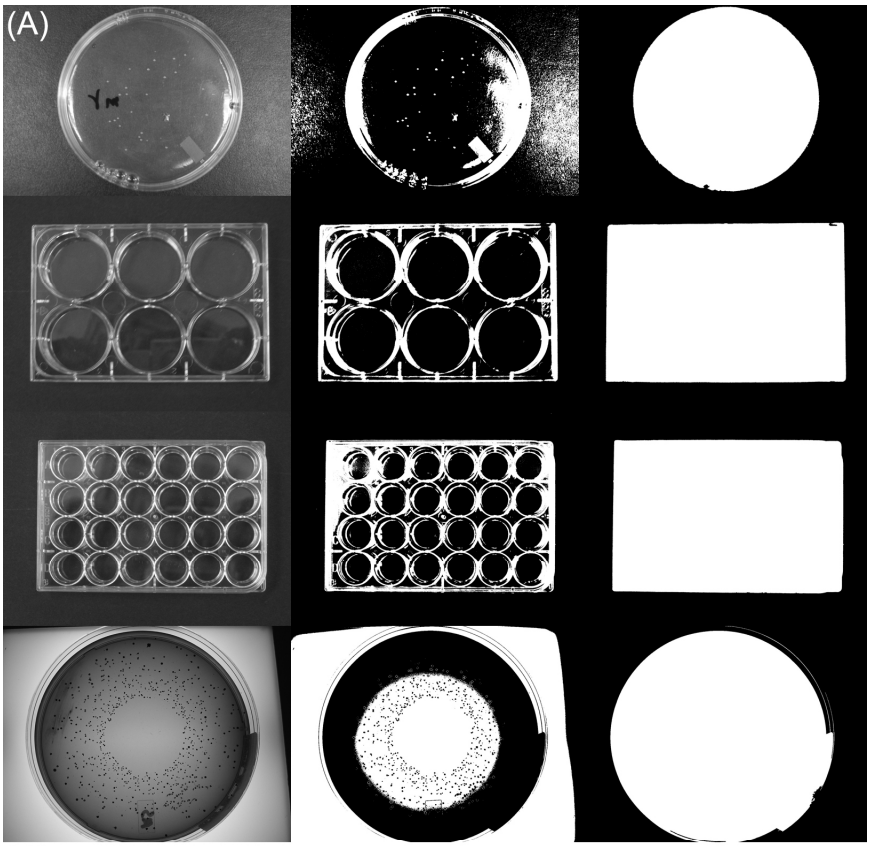
Additionally, Petri dishes with two different types of medium and bacteria strains are used in our experiments. The first type of images is obtained from the Department of Pediatric Dentistry at the University of Alabama at Birmingham. This type of plate contains blue color Mitis-Salivarius agar which is used for isolating *Mutans Streptococci*. These acid-producing bacteria are commonly seen in our oral cavity, and have been implicated as a major etiological agent that 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 plates contains the clear LB agar which is widely used in laboratories for growing *Escherichia Coli*.

Dish/Plate Region Detection

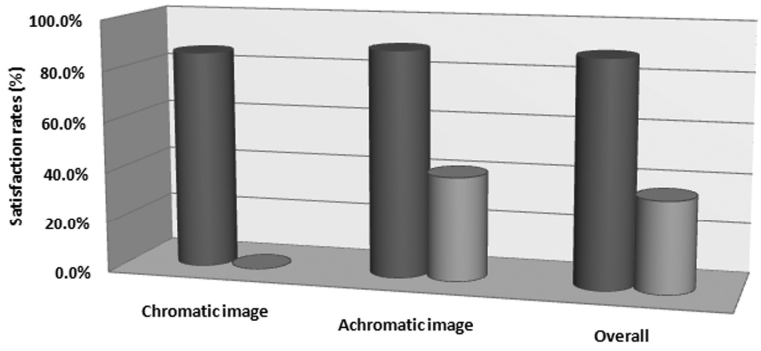
In this experiment, we compare the proposed dish/plate detection algorithm with Otsu's segmentation algorithm. Some sample segmentation results are demonstrated in Figure 3 (A). We evaluate the performance of the proposed dish/plate detection algorithm and Otsu's method by applying both algorithms on 300 images, including 36 chromatic images and 264 achromatic images. The performance is measured by the satisfaction rate which is defined as below:

$$\frac{\text{Number of images that dish/plate regions are correctly detected}}{\text{Total number of images}} \times 100\% \quad (2)$$

The overall satisfaction rates for the proposed method and Otsu's method are 89.0% and 36.3%, respectively. For the 36 chromatic images, the satisfaction rates for the proposed method and Otsu's method are 86.1% and 0%, respectively. For the 264 achromatic images, the satisfaction rates for the proposed method and Otsu's method are 89.4% and 41.3%, respectively. It is obvious that the proposed method outperforms Otsu's method in dish/plate region detection. We summarized the satisfaction rates for both methods in Figure 3 (B).



(B)



	Chromatic image	Achromatic image	Overall
Proposed method	86.1%	89.4%	89.0%
Otsu's method	0.0%	41.3%	36.3%

Figure 3. Performance comparison for dish/plate region detection

Colony Recognition

In this Section, the performance of this system on colony recognition is evaluated. It is more reasonable to discuss the counter performance on chromatic and achromatic images separately in this experiment since the characteristics of them are quite different. We compared the proposed

counter (P.C.) with the Clono-Counter (C.C.) which is reported by Niyazi in 2007 (Niyazi et al., 2007). We apply both counters on 10 chromatic images and 30 achromatic images. The enumeration results are then compared with the ground truth for calculating the precision, recall, and F-measure values.

For chromatic images, the precision values of the P.C. and C.C. methods are 0.97±0.04 and 0.56±0.24, respectively; their recall values are 0.89±0.07 and 1.00±0.01, respectively; their F-measure values are 0.92±0.03 and 0.69±0.19, respectively. Table 1 shows the performance comparison on chromatic images.

To evaluate the robustness of the proposed counter (P.C.) on achromatic images, we conduct the following two experiments, and compare the performance of P.C. with that of C.C.

In the first experiment, we test the proposed counter (P.C.) on 30 achromatic images. The performance of the P.C. and C.C. methods on these achromatic images are summarized in Table 1. From Table 1, we can observe that the P.C. significantly outperforms the C.C. method. The average precision, recall, and F-measure values of the P.C. method are 0.69±0.30, 0.87±0.09, and 0.72±0.18, while the corresponding values of C.C. are 0.00±0.00, 0.00±0.00, and 0.00±0.00, respectively.

Table 1. Performance comparison on chromatic and achromatic images

Image Type	Method	Precision	Recall	F-measure
Chromatic	P.C.†	0.97±0.04	0.89±0.07	0.92±0.03
	C.C.‡	0.56±0.24	1.00±0.01	0.69±0.19
Achromatic	P.C.†	0.69±0.30	0.87±0.09	0.72±0.18
	C.C.‡	0.00±0.00	0.00±0.00	0.00±0.00
Overall	P.C.†	0.76±0.28	0.88±0.08	0.77±0.18
	C.C.‡	0.14±0.27	0.25±0.44	0.17±0.31

In the second experiment, we further apply the proposed method on 30 different achromatic images taken from the same dish, but with different background surfaces, zooms, and lighting conditions. We measure the precision, recall, and F-measure of the proposed counter. The average precision, recall, and F-measure on the 30 achromatic images are 0.95±0.04, 0.80±0.04, and 0.87±0.02, respectively. The results of the consistency analysis show the proposed system is quite consistent since the variance of enumeration results from the same dish is small.

Colony Separation

In recognizing colonies, there are some clustered colonies that need to be further divided into separate colonies. As mentioned earlier, we adopt the Watershed algorithm to solve this problem and found it effective in splitting clustered colonies according to our experimental results. An example of the splitting result with the use of Watershed algorithm is given in Figure 4.

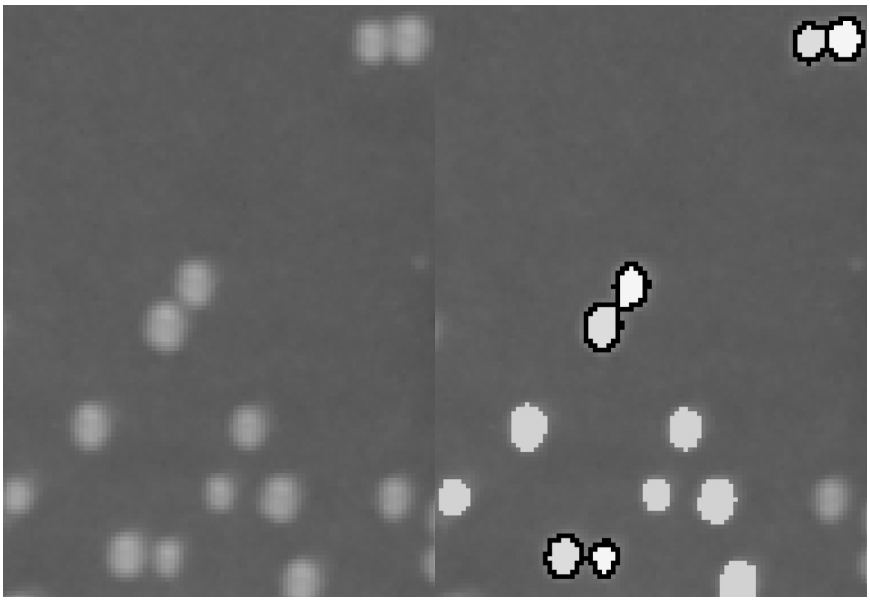


Figure 4. An example of colony separation

In our experiment, we checked the performance of the Watershed algorithm on 19 randomly selected segments with clustered colonies which actually 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 mentioning that the Watershed algorithm is an integral part of the proposed system, where each step contributes to the better performance of the subsequent steps.

DISCUSSIONS AND CONCLUSIONS

In this chapter, we introduce a robust and effective automated bacterial colony enumeration system. The proposed system has the ability to identify various types of dish/plate in images, and then recognize colonies in the identified dish/plate region. The morphology of the colony segment is checked for distinguishing clustered colonies from single colonies. To obtain the accurate number of colonies, the Watershed transform is then applied to separate the aggregated colonies. After these steps, the number of colonies can be enumerated as the total number of segments in the image.

The most challenging part in this study is to handle achromatic images, since colonies look very similar to the clear medium (background). In addition, there exist a lot of noises on the plate such as bubbles, small scratches, and small markers. These round-shaped objects also look very similar to colonies and it is often hard to tell whether or not they are colonies even by human eyes. This makes the colony isolation task extremely difficult. The experimental results show the proposed system addresses these challenges and demonstrates reasonable performance on both chromatic and achromatic images.

It is worth mentioning that this study has the following contributions. First, the proposed counter can handle various kinds of dish/plate, including round and rectangular shaped dishes/plates,

which is a very desirable feature. Second, we use general digital camera images as its input, and there is no need to purchase expensive and dedicated image acquiring devices. The third contribution is that our counter can distinguish chromatic from achromatic images and process both color and clear medium. In addition, since our counter is a fully automated, software-centered colonies counter, all the user needs to do is to take the pictures of the dishes/plates and leave the remaining job to the counter. Users are no longer involved in the tedious and time-consuming process of selecting the target area or providing values for parameters. Moreover, the performance of the proposed counter is promising for both chromatic and achromatic images. The above desirable features make this enumeration system very flexible and attractive to laboratories. Laboratories can save precious time and allocate limited budget on more important tasks.

Though the performance of our proposed bacterial colony enumeration system is very promising, there are still some remaining issues. In the future, we will put more effort on distinguishing the colony from noises in achromatic images. We may introduce more colony features such as color, texture, and topology to improve colony recognition. Another issue in this study is that bacterial colonies may have different shapes as well as colors in the real world; however, currently we can only handle clustered colonies which are composed of round-shaped colonies with Watershed algorithm. The ultimate goal is to accurately differentiate various bacteria species grown in the same dish and correctly enumerate the colonies for each bacteria species. We believe it will have great benefit for clinical dental studies.

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KEY TERMS

Bacterial Colony Counter

A manual, semi-automatic or automatic instrument used to enumerate the number of bacterial colonies on a plate.

Colony Forming Unit

Colony Forming Unit (CFU) is a measure of the number of viable microbes in microbiology. The theory behind is a single living microbe can grow and form a colony via binary fission.

Contrast Limited Adaptive Histogram Equalization (CLAHE)

Contrast Limited Adaptive Histogram Equalization is a contrast enhancement method that performs on grayscale image. This method operates on small regions, called tiles, in the image,

rather than the entire image. The contrast of each tail is enhanced by adopting histogram equalization.

HSV Color Space

HSV stands for Hue-Saturation-Value, which describe colors as points in a cylinder whose central axis ranges from black to white with neutral colors between them, where angle around the axis corresponds to “hue”, distance from the axis corresponds to “saturation”.

Image Segmentation

A process that partitions images into meaningful regions based on certain characteristics or properties such as intensity, color, shape, and/or texture.

Otsu’s Method

A thresholding technique used to determine an optimal threshold value for dividing data into two classes such that the intra-class variance is minimized and the inter-class variance is maximized.

Watershed Transformation

A morphological segmentation method that partitions images based on the gradient regardless of the shape and size of the object.