An Automated Bacterial Colony Counting System

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Abstract

Bacterial colony enumeration is an essential tool for many widely used biomedical assays. However, bacterial colony enumerating is a low throughput, time consuming and labor intensive process since there might exist hundreds or thousands of colonies on a Petri dish, and the counting process is often manually performed by well-trained technicians. In this paper, we introduce a fully automatic yet cost-effective bacterial colony counter. Our proposed method can recognize chromatic and achromatic images and thus can deal with both color and clear medium. In addition, our proposed method can also accept general digital camera images as its input. The whole process includes detecting dish/plate regions, identifying colonies, separating aggregated colonies, and finally reporting consistent and accurate counting results. Our proposed counter has a promising performance in terms of both precision and recall, and is flexible and efficient in terms of labor- and time-savings.

Keywords:

Biomedical image mining, colony counting.

1. Introduction

In biomedical research and clinical diagnosis, there is a great need to quantify the amount of bacteria in the samples. To analyze the result from bacterial culture, bacterial colony enumeration is used to count the number of viable bacteria as colonies. This type of assays 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 to form colonies (a.k.a. colony forming unit - CFU) on the plates. The evaluation is done by examining the survival rate of microbes in a sample. These assays are also widely used in biomedical examinations, food and drug safety test, environmental monitoring, and public health [1].

However, bacterial colony enumerating is a low throughput, time consuming and labor intensive process since there might exist hundreds or thousands of colonies on a Petri dish, and the counting process is often manually performed by well-trained technicians. 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 [2]. Thus, having consistent criteria is very important.

To reduce the operator's workload and to provide consistent and accurate results, colony counting devices were developed and commercialized in the market [3]. We reviewed these counters available on the market and classified them into two categories.

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

The second type of counter is semi-automatic or automatic counters which are often very expensive. These high-priced devices often come with their own image capture hardware for acquiring high quality images to optimize the counter's efficiency and performance. However, the affordability of this kind of equipment is still a non-trivial issue for most laboratories due to the high price of such equipment in the market. Some laboratories that need to perform a huge amount of enumeration tasks may require more than one high-throughput counter to fit their needs.

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Thus, colony enumeration devices pose a significant budgetary challenge to many laboratories [5].

In addition, some automatic counters accurately detect colonies by growing bacteria on special growth medium which contains fluorogenic substrates [8]. Bacteria metabolize the substrates, and then produce fluorescent product for detection. These systems are extremely sensitive, and are good for detecting microcolonies. However, the fluorogenic substrates used in the medium are costly, and the fluorescence can only be detected by using a sensitive instrument. Besides, some automatic 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/medium that differ from their default settings. In such cases, human operators are heavily involved in the operation, and it is thus not efficient for high throughput processing of plates/dishes.

Further, laboratories have needs to use various types of dishes and plates in examinations. However, most of the commercial counters are designed for measuring 60-150mm Petri dish, thus, lack the flexibility for accommodating plates with different sizes and shapes.

In addition to the above problems, some counters use only binary images for detecting colonies. Plenty of important characteristics of the colony, such as color, are lost although they can be used to identify the genus of the bacteria.

To address the above problems, our goal in this study is to design and implement an inexpensive, software-centered system for detecting bacterial colonies in a fully automatic manner. Thus, more time and money can be allocated to other priorities for those laboratories.

Nowadays, image acquiring devices such as digital cameras and flatbed scanners, become more popular and affordable. Hence, it motivates us to use these devices to obtain cost-effective yet high-quality images for colony counting.

In automating the bacteria colony counting process, one of the challenges is that the colors of bacteria colony and culture medium vary. This is because different strains of bacteria may require different nutrients, and these ingredients make the culture medium with various colors. In addition, bacteria grow on different kinds of culture medium may appear in different color. Hence, while some bacteria colony images contain abundant color information, some do not. For example, *Mutans Streptococci* appears as black colonies on the blue color Mitis-Salivarius agar, and *Escherichia Coli* appears as white colonies on the clear/transparent LB agar. Based on our experience in using existing software for colony counting, there is no single best algorithm that can satisfy the needs of different types of medium. Hence, we believe it is more appropriate to process images that do or do not carry color information separately.

Although human operators can easily recognize bacteria colonies on medium after some training, computers can hardly "see" these colonies without any prior knowledge. This motives us to design a three-step approach for simulating human's recognition behavior. When a human operator examines a bacteria colony image, he gradually identifies objects from the image. First, the dish/plate region, the largest object in the image, is identified. Second, within the dish/plate region, human starts to find colonies based on some criteria such as color and shape. An illustration of this hierarchy is shown in Figure 1. If colonies are clustered together, the operator will try to separate the clustered colonies based on his best visual judgment. Once all colonies have been identified, the operator counts the total number of colonies.

In this paper, the proposed framework differentiates the processing of colony images with rich color information from that of those with little color information. It first determines whether or not an image carries color information. Then, it locates the dish/plate area. In the third step, possible colony candidates are identified which are subject to further statistics test in order to identify the 'true' colonies. Clustered colonies are further separated by Watershed algorithm [7]. Finally, the number of remaining segments is reported back as the total colony count.

In the remaining of this paper, we introduce the system details in Section 2. Section 3 demonstrates experiment results. Section 4 concludes the paper.

Figure 1. The hierarchical structure of objects in bacteria colony images

2. The proposed bacterial colony counter

The overview of the proposed system is illustrated in Figure 2. First, for an input image, we examine its chromatic/color components, and a proper processing method is selected, depending on what type of image

(chromatic/achromatic) it is. In the next step, we gradually extract objects from the image step-by-step based on their hierarchical structure as illustrated in Figure 1.

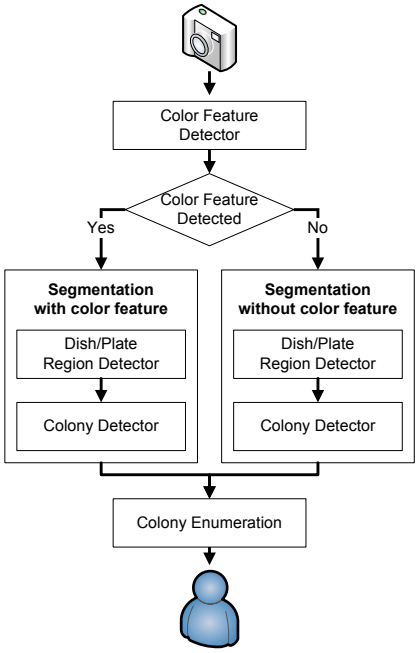

Figure 2. The overview of the proposed system

Once all the colonies on the image are identified, we check the morphology of each segment. This is necessary because some colonies may aggregate together to form a large cluster. Hence, to obtain an accurate colony count, those clustered colonies need to be separated. For this purpose, we adopt the Watershed algorithm [7] to detect and segment those plausible colony clusters. Once all the colonies on the dish/plate have been identified and isolated, we simply count the number of detected segments and use it as the total count of bacteria colonies.

2.1. Color feature detection

As mentioned in Section 1, some bacteria colony images may contain abundant color information. For those colored images, we propose a color feature based method to detect foreground objects in the target region (region-of-interest). On the other hand, those images with very little color information (almost no hue) shall be dealt with in a different way.

To choose a proper method for different types of images, we first need to determine whether the imported image is chromatic or achromatic. The checking is achieved by examining the standard deviation of mean values from each color channel R, G, and B. This is based on the fact that if the RGB values of a pixel are close to each other, it is most likely a gray pixel, and vice versa. Thus, a small standard deviation indicates low hue or lack of chromatic components. The smaller the standard deviation is, the higher the possibility that the image is achromatic (e.g. those colony images with clear medium and white colonies.) It is at this point that chromatic images (e.g. *Mutans Streptococci* appears as black colonies on the blue color Mitis-Salivarius agar) are distinguished from achromatic images (e.g. *Escherichia Coli* appears as white colonies on the clear LB agar), which will be dealt with separately.

In view of the different characteristics of achromatic and chromatic images, we develop different methods for these two types of images in the subsequent image segmentation step.

2.2. Image segmentation

The core of the proposed bacterial colony counter is image segmentation. The goal of segmentation is to distinguish foreground objects from the background. For this purpose, there are two popular choices of techniques, namely the thresholding techniques and the clustering techniques. The thresholding techniques use a global threshold value to separate foreground and background, and the clustering techniques partition objects based on their inter- and intra-class similarities. The thresholding techniques are quite straightforward and efficient, but are not stable when dealing with images containing more than two classes. According to our preliminary experiments, the performance of multi-class clustering methods, which are more complicated and time-consuming, are generally worse than that of the thresholding techniques in terms of robustness, explainability, and projectibility. In this paper, we propose a new thresholding based technique for bacterial colony image segmentation.

To do segmentation with thresholding techniques, we have to solve the problem that the target region contains more than two classes. The natural hierarchical structure of the objects in colony images (as shown in Figure 1) indicates that we may be able to gradually separate them in a progressive way.

Our target, at the first level, is the entire image. The foreground object is the dish/plate region, and the background is the area surrounding the dish/plate region. After the dish/plate area is separated, we move to the second level in which the foreground objects are colonies and the background includes medium and other artifacts within the dish/plate region.

As aforementioned, we propose different approaches to deal with images with chromatic and achromatic medium separately in the subsequent processes. It is much easier to perform segmentation on chromatic images than achromatic images since they contain more color information. In the following discussions, we first introduce how to perform segmentation on chromatic images, then the approach for achromatic images.

2.3. Segmentation on chromatic images

In this step, our goal is to identify the dish/plate region in an image, and then, recognize colonies in the detected dish/plate region. The motivation is to reduce the operator's workload by eliminating the process of manually specifying the target dish/plate region. To distinguish the dish/plate region from the background, we first perform the contrast limited adaptive histogram equalization (CLAHE) on the converted grayscale images which operates on small regions called tiles in the image rather than the entire image [6]. Each tile's contrast is enhanced and the neighboring tiles are then combined using bilinear interpolation to eliminate artificially induced boundaries.

Then we apply the Otsu's method [9] on the contrast enhanced image to identify the dish/plate region as a target region. For some target regions detected this way, there may be small holes inside, and we fill in the holes by adopting a morphology-based method and consolidate the target regions.

Sometimes, this method can also detect some smaller objects that are not part of the target dish/plate region. We assume the target region should occupy the majority (and central) part of the image, thus there is an extra step in our algorithm which is designed to remove those isolated small objects. A few of detected target regions of dish/plate, after applying the above steps, are shown together with their original images in Figure 3.

The results show that the automatic dish/plate region detection algorithm is effective regardless of the size and shape of the dish/plate. After the dish/plate region has been extracted, we can apply the segmentation again on the detected dish/plate region.

The second step is to isolate colonies on the dish/plate, identify clustered colonies, and separate aggregated bacteria colonies for subsequent colony enumeration. In addition to using Otsu's method [9] to separate colonies and medium, we also adopt color similarity in HSV (Hue-Saturation-Value) color space to assist the colony boundaries detection [10]. This is necessary because a simple global threshold cannot correctly identify all colonies due to the existence of artifacts such as scratches, dusts, markers, bubbles, reflections, and dents in the image. The calculation of color similarity in HSV color space is shown in Equation 1.

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

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.

This is based on the assumption that pixels inside a segment, no matter it is a colony segment or a medium segment, have higher similarity values with its neighboring pixels, and pixels along the segment boundary have lower similarity values with their neighbors. 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 in the same segment have higher minimum similarity values. On the contrary, pixels on the boundary of a segment have lower values. After the calculation, the boundaries are more evident, and the minimum color similarity values formed a matrix as a grayscale image. Thus, we can adopt the Otsu's method used in the dish/plate region detection stage to further distinguish background (medium areas) from foreground objects (candidate colonies).

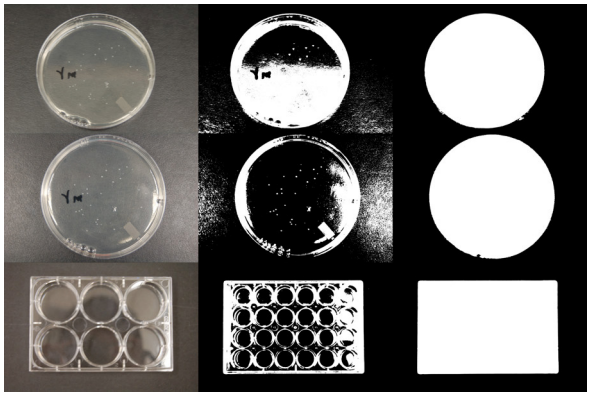

Figure 3. Segmentation results for detecting dish/plate regions. Raw images (left column); Otsu's method (middle column); proposed method (Right column)

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. There is a need to split them in order to get the correct colony counts. To separate the connected colonies, we consider the intensity gradient image as a topological surfaces [7], thus the watershed algorithm can be applied to divide clustered colonies in the image just as water flood in a topographical surface. To illustrate the concept, we demonstrate the application of watershed algorithm in Figure 4.

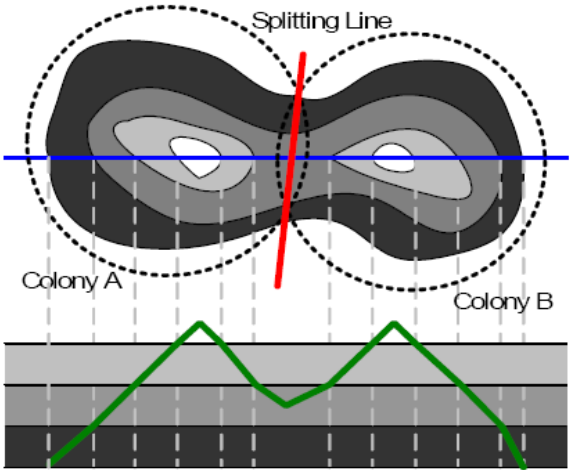

Figure 4. The concept of watershed algorithm

After applying the watershed algorithm, almost all clustered colony segments can be separated and identified and are ready for the colony enumeration.

2.4. Segmentation on achromatic images

The most challenge 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. Besides, 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, our method is also based on the hierarchical structure of objects aforementioned. In the first step, the dish/plate region can be detected by using the same approach as described in Section 2.3. In the colony detection stage, we develop a different method to alleviate the low contrast and artifacts problems. We also apply Otsu's method to isolate colonies. However, Otsu's method is much less accurate on achromatic images due to the presence of artifacts. An additional noise removal step is performed for those achromatic images.

The color similarity as described in Section 2.3 cannot be applied since achromatic images lack color information. In this paper, we propose 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 large-size 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 second step is to remove those small artifacts which are very similar to the colonies in the dish/plate. In this step, area size is not a good determinant since the area size range of those small artifacts is about the same as 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 the distribution of colonies pixels. Those small artifacts belong to background distribution; however, they have overlapped with the colony distribution. Therefore, we assume that colonies should have significantly different intensity values than their surrounding background, and it is highly possible 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, by the $\alpha = 0.01$.

After excluding most of the artifacts, we apply the watershed algorithm to separate clustered colonies as described in Section 2.3.

2.5. Colony enumeration

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

3. Experimental results

In our experiments, we use four different digital cameras as the image acquiring devices to obtain

dish/plate images for bacterial colony detection. The four 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, 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 of 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 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 *Escherichia Coli* culture.

3.1. Dish/Plate detection

In this experiment, we compare the proposed dish/plate detection algorithm with Otsu's method. Some sample segmentation results are demonstrated in Figure 3. In addition, we also evaluate the performance of the proposed dish/plate detection algorithm and Otsu's method by applying both methods on 100 chromatic and achromatic images. The satisfaction rates for the proposed method and Otsu's method are 96% and 38%, respectively. For the 25 chromatic images, the satisfaction rates for the proposed method and Otsu's method are 92% and 0%, respectively. For the 75 achromatic images, the satisfaction rates for the proposed method and Otsu's method are 97% and 50%, respectively. It is obvious that the proposed method outperforms Otsu's method in dish/plate region detection.

3.2. Colony detection

Since the characteristics of the chromatic and achromatic images are quite different, it is more appropriate to discuss the counter performance on them separately. In the experiments, we compared the proposed counter (P.C.) with the Clono-Counter (C.C.) [4] which is reported by Niyazi in 2007, and the automatic counter (A.C.) proposed in our previous study [11]. For chromatic images, the precision values of the A.C. and C.C. methods are 0.97±0.03 and 0.52±0.19, respectively; their recall values are 0.96±0.04 and 0.99±0.01, respectively; their F-measure values are 0.96±0.01 and 0.67±0.18, respectively. The precision, recall, and F-measure values of the proposed counter (P.C.) are about the same as that of the A.C. method 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 A.C and C.C.

In the first experiment, we test the proposed counter (P.C.) on 24 achromatic images (9 images with good quality and 15 images with poor quality). The performance of the P.C., A.C., and C.C. methods on good/poor quality images are summarized in Table 1. From Table 1, we can observe that the P.C. significantly outperforms the A.C. and C.C methods. The average overall precision, recall, and F-measure values of the P.C. method are 0.61±0.29, 0.94±0.06, and 0.69±0.20, while the corresponding values of P.C. and C.C. are (0.44±0.24, 0.68±0.24, 0.44±0.13) and (0.00±0.00, 0.00±0.00, 0.00±0.00), respectively.

In our second experiment, we further apply the proposed method on 15 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 15 achromatic images are 0.93±0.11, 0.87±0.04, and 0.90±0.07, respectively. The results of the consistency analysis show the proposed system is quite consistent.

Table 1. Performance comparison on achromatic images

Image Condition	Method	Precision	Recall	F-measure
Good Quality (9)	P.C.	0.94 ± 0.07	0.88 ± 0.02	0.90 ± 0.03
	A.C.	0.71 ± 0.06	0.42 ± 0.16	0.52 ± 0.12
	C.C.	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00
Poor Quality (15)	P.C.	0.41 ± 0.16	0.98 ± 0.04	0.56 ± 0.13
	A.C.	0.27 ± 0.12	0.84 ± 0.07	0.40 ± 0.12
	C.C.	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00
Overall	P.C.	0.61 ± 0.29	0.94 ± 0.06	0.69 ± 0.20
	A.C.	0.44 ± 0.24	0.68 ± 0.24	0.44 ± 0.13
	C.C.	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00

P.C. : The proposed counter
A.C. : Automatic counter [11]
C.C. : Clono-Counter [4]

3.3. Splitting clustered colonies

In the process of detecting colonies, there exist some clustered colonies that need to be further divided into separate 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 result of Watershed algorithm in Figure 5.

In our experiment, we checked the performance of the Watershed algorithm on 19 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.

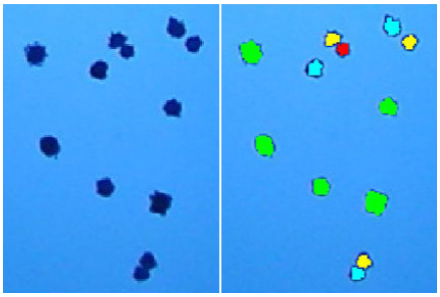

Figure 5. Clustered colonies split by watershed algorithm

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.

4. Discussions, Conclusions, and Future Work

In this paper, we introduce a robust and effective automatic bacterial colony counter with the ability to recognize chromatic and achromatic images, detect the dish/plate regions, isolate colonies on the dish/plate, and further, separate the clustered colonies for accurate counting of colonies. The proposed counter has the following contributions.

First, our proposed method can handle various kinds of dish/plate, including circular and rectangular shaped dish/plates. Second, it can accept general digital camera images as its input. The third contribution is that our proposed method can recognize chromatic and achromatic images and deal with both color and clear medium. The most challenging part in this study is to handle clear medium images, since colonies look very similar to the background. There also exists a lot of noise on the plate such as bubbles, small scratches, and small markers. Some round-shaped small objects are very similar to the colonies and sometimes it is hard to distinguish them from real colonies even by trained human eyes. This makes the colony isolation task extremely difficult. In this paper, we address those challenges and demonstrate a reasonable performance in both color and clear medium images.

The above features also make our proposed method very flexible and attractive to laboratories. In addition, our proposed counter operates automatically without any human intervention, and the performance is very quite promising for both color and clear medium.

In the future work, we plan to detect and distinguish different species of bacteria from a single colony dish/plate. Ultimately, our goal is to accurately classify different kinds of bacterial colonies and produce the correct count for each class, which could greatly benefit clinical studies.

5. Acknowledgement

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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., pp. 474-485, 1994.

[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.

[10] G. A. F. Seber, “Multivariate Observations”, Wiley, 1984.

[11] C. Zhang and W.-B. Chen, “An Effective and Robust Method for Automatic Bacterial Colony Enumeration,” in Proc. of the IEEE Intl. Workshop on Semantic Computing and Multimedia Systems, in conjunction with the 2007 Intl. Conf. on Semantic Computing, pp. 581-588, September 17-19, Irvine, CA, USA, 2007.