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Automated counting of bacterial colonies on agar plates based on images captured at near-infrared light

Guozhen Zhu[a], Bin Yan[b], Mengting Xing[a], Chunna Tian[a,*]

[a] School of Electronic Engineering, Xidian University, Xi'an 710071, China
[b] College of Mechanical and Electronic Engineering, Northwest A&F University, Yangling, Shaanxi 712100, China

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ABSTRACT

Counting colonies is usually used in microbiological analysis to assess if samples meet microbiological criteria. Although manual counting remains gold standard, the process is subjective, tedious, and time-consuming. Some developed automatic counting methods could save labors and time, but their results are easily affected by uneven illumination and reflection of visible light. To offer a method which counts colonies automatically and is robust to light, we constructed a convenient and cost-effective system to obtain images of colonies at near-infrared light, and proposed an automatic method to detect and count colonies by processing images. The colonies cultured by using raw cows' milk were used as identification objects. The developed system mainly consisted of a visible/near-infrared camera and a circular near-infrared illuminator. The automatic method proposed to count colonies includes four steps, i.e., eliminating noises outside agar plate, removing plate rim and wall, identifying and separating clustered or overlapped colonies, and counting colonies by using connected region labelling, distance transform, and watershed algorithms, etc. A user-friendly graphic user interface was also developed for the proposed method. The relative error and counting time of the automatic counting method were compared with those of manual counting. The results showed that the relative error of the automatic counting method was −7.4%~ + 8.3%, with average relative error of 0.2%, and the time used for counting colonies on each agar plate was 11–21 s, which was 15–75% of the time used in manual counting, depending on the numbers of colonies on agar plates. The proposed system and automatic counting method demonstrate promising performance in terms of precision, and they are robust and efficient in terms of labor- and time-savings.

1. Introduction

Counting bacterial colonies on agar plates is a mostly used method to estimate the concentration of live bacteria in culture. It is widely used in food and drug safety test, biomedical examinations, environmental monitoring, and public health (Liu et al., 2004). However, counting bacteria on agar plates is usually performed by technicians manually. This manual enumeration process is subjective, error prone, low throughput, time-intensive, tedious, and laborious since up to 300 colonies on an agar plate would need to be counted (Chang et al., 1994; Brugger et al., 2012). Therefore, some automatic counting methods with high efficiency which could replace manual counting method are desirable. At present, a kind of automatic digital bacterial colony counter is widely used in most laboratories. However, it is not truly automatic since it depends on persons who use probe to identify each colony so that the sensor system can sense and register each count. Although it is generally a straightforward task, but can become very laborious and time-consuming when many plates/dishes have to be enumerated.

With the development of computer vision technology, various kinds of automatic analysis systems used to count colonies based on image processing and pattern recognition have been developed (Ates and Gerek, 2009; Chen and Zhang, 2009; Lawless et al., 2010; Men et al., 2008). Chen and Zhang (2009) introduced a fully automatic yet cost-effective bacterial colony counter which can not only count but also classify colonies. Brugger et al. (2012) implemented a colony counting system to discriminate bacterial colonies from blood and other agar plates. A time-lapse shadow image analysis method, involving capturing images of Petri dish and analyzing the images, was proposed by Ogawa et al. (2012). Hu (2013) applied distance transform and progressive erosion methods to realize counting and identifying cell colonies automatically. A Colonyzer, developed by Lawless et al. (2010),

* Corresponding author.
E-mail address: chnatian@xidian.edu.cn (C. Tian).

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is capable of collecting images and doing image analysis on the size, granularity, color, and location of micro-organism cultures grown on solid agar. Other solutions have also been put forward to estimate the number of colonies on agar plates by using different methods (Chiang et al., 2015; Ferrari et al., 2017; Matic et al., 2016). Moreover, some software run on computers have been developed to count colonies, such as NICE (NIST's Integrated Colony Enumerator) (Clarke et al., 2010), CHiTA (Circular Hough Image Transform Algorithm) (Bewes et al., 2008), OpenCFU (Geissmann, 2013), ImageJ (Cai et al., 2011), ImageJ macro Cell Colony Edge and CellProfiler Pipeline Cell Colony Counting (Choudhry, 2016), and MicroCount (Siqueira and Carvalho, 2017). An Android mobile cross-platform open source software for automatic bacteria colony counting has also been developed (Minoi et al., 2016).

In these studies, traditional cameras were used to capture images with colonies at visible light. In this situation, most colony detection methods function adequately only when the colonies have a relatively high contrast between bacteria colonies and background, when the colonies are large and well spaced, and when the images are evenly illuminated. If these optimal conditions are violated, the accuracy is lost (Choudhry, 2016; Frost et al., 2016). To investigate whether the light wavelength have influence on bacteria counting accuracy, Yoon et al. (2015) applied hyperspectral imaging technology in the visible and near-infrared (NIR) spectral range from 400 nm to 1000 nm to realize colony counting. They found that the colony morphology including size and texture were dependent on wavelength of visible light, but at the near-infrared light of 850 nm, the colony morphologies were clear and had obvious contrast with background (Yoon et al., 2015). Masschelein et al. (2012) also developed a colony counting system using hyperspectral imaging, and they showed that colony counting could be automatic through automatic recovery of illuminant power spectrum and reflectance. Although hyperspectral imaging technology could give high efficiency, the instrument used is expensive, usually higher than 100,000 USD dollars. However, up to now, no reports have been found on developing a cheap colony counting system at near-infrared light.

Raw milk is the source of dairy products. All raw milk produced and processed in legal dairy plants must be submitted periodically for analysis on total bacterial count. Therefore, raw cows' milk was used to cultivate bacteria in this study. A system with near-infrared light as light source was constructed, and an automatic method was put forward to detect and count colonies on agar plates by processing images obtained at near-infrared light in this study. Hope the study to provide a convenient and cost-effective system which could obtain images with obvious and stable contrast between colonies and background, and to offer an automatic colony counting method to save labor and time.

2. Materials and methods

2.1. Bacterial cultures

In this study, raw milk, produced by 'Holstein' cows, were used to culture bacteria according to the Chinese National Standard of GB 4789.2-2016 (2016). The preparation procedure used in culturing bacteria is introduced here. According to the ratio of 2.5 g agar powder, produced by Aoboxing Bio-technology Co., Ltd., Beijing, China, in 100 ml deionized water to prepare culture medium. One ml of raw milk sample was diluted in 9 ml of sterilized saline to obtain the milk solution with dilution degree of 10^{-1}. Then 1 ml of diluted milk was diluted again to obtain milk solution with dilution degree of 10^{-2}. According to this method, decimal dilutions up to 10^{-8} were prepared. From 10^{-5} to 10^{-8}, 1 ml diluted milk at each dilution degree was transferred to sterilized agar plate in diameter of 90 mm. Then, 20 ml of prepared culture medium at 46 °C was put into the plate. After being blended and solidified, the prepared samples were immediately incubated at 37 °C for 24 h in an incubator (SPX-250, Jiangnan Instrument Factory, Ningbo, China). Triplicate was done for each dilution degree.

Breed and Dotterrer (1916) suggested that to ensure statistical accuracy, the number of colonies on an agar plate was limited to no fewer than 30 and not excessively high so as to not compromise visual identification. Chinese National Standard of GB 4789.2-2016 (2016) regulates that if the number of colonies in a plate is fewer than 30 or more than 300, the cultivation at this dilution is regarded as unqualified. Tomasiewicz and Peeler (1980) also suggested that for manual counting, in general, the range in common acceptance for countable number of colonies on a 100 mm agar plate is between 30 and 300. Therefore, if the estimated amount of bacteria colonies in an agar plate was between 30 and 300, the bacteria colonies in the plate were enumerated manually by three trained persons using a colony counter (XK97-A, Bangxi Instruments Technology Co., Ltd., Shanghai, China). The mean of triplicate was used as the result. Finally, 34 qualified samples, whose colonies were more than 30 and less than 300, were obtained in this study.

2.2. Near-infrared image collection system

Obtaining images with obvious and stable contrast between colonies and background is very important to improve colony detection accuracy. Since the colonies had obvious contrast with background at 850 nm (Yoon et al., 2015), an image data collection system at near-infrared light was constructed in this study, shown in Fig. 1. It consisted of a grayscale visible/near-infrared camera (model BFLY-PGE-13E4M-CS, PointGrey Corporation, Canada) with response spectra range of 300–1100 nm and 1.3 million effective pixels, a circular near-infrared illuminator (VR180-75-IR, Shanghai Vanch Optics Sci-Tech Ltd., Shanghai, China) at 860 nm with inner diameter of 146 mm, a power supply unit for near-infrared illuminator, a supporter with platform in black color, agar plate with colonies to be counted, a personal computer, and a dark box. The lens of the camera was concentric to the circular illuminator and the agar plate. The assembly height of the camera was about 150 mm over the agar plate. The camera was connected to the computer via a USB data line. Except for the computer and power supply unit, other parts of the system were put in the dark box to avoid any influence of environment light on images.

2.3. Procedures

Previous study showed that when the agar plates with colonies were placed upside down on the platform, the obtained images were clear and the colonies had obvious contrast with the background. Therefore, the agar plates with colonies were placed upside down on the platform in the study. The centers of the agar plate, circular illuminator, and lens of the camera should be concentric to make the image of the agar plate without distortion. Before capturing, the focus of the lens was adjusted to make only agar plate be in the image as far as possible. The output power of the power supply was set to 2 W, which can make the colonies have obvious contrast with the background. To assess accuracy and performance of the developed automated colony method, after the image of the agar plate was captured using the developed image collection system, routine manual counting was performed by 3

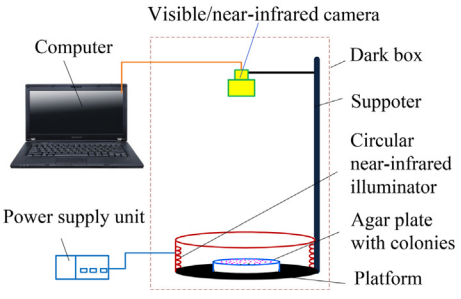

Fig. 1. Schematic diagram of the used near-infrared image collection system.