# A Novel Methodology for Automatic Bacterial Colony Counter

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## ABSTRACT
Many biological procedures depend on an accurate count of the bacterial colonies and other organisms. In biomedical research and clinical diagnosis, there is a great need to quantify the amount of bacteria in the samples.This paper presents a simple and cost effective methodology for automatically counting the Bacterial Colonies (BC). The proposed methodology for automatic colony counter is based on digital image processing techniques. Proposed methodology is tested with different type of filter images. It is observed that the results obtained with the proposed counter were not significantly different from the manual counting.

## General Terms
Digital image processing, Pattern Recognition

## Keywords
Adaptive median filter, bacterial colony, bacterium, colony forming unit, microorganisms, morphological image processing.

## 1. INTRODUCTION
If we go through the literature we will found that Bacterial colony (BC) counting plays an important role to check the effectiveness of disinfectants, for conducting assays not only in medical examinations, but also in food and drug safety evaluations in pharmaceutical industries and hospitals. Bacterial colony counting is also used in microbiology labs to test retention efficiency of filters or membranes.

Automation of colony counting has been of increasing interest for many decades, and these methods have been shown to be more consistent than manual counting. It is also found that automated colony counts had significantly less variation when reanalyzing plates than those manually determined by individual or multiple observers [1]. Whether it is manual counting or automatic counting, to make the colonies more visible, triphenyle tetrazolium chloride (TTC) was incorporated. Live bacteria convert TTC to a product with a deep red colour, and the contrast of colorized bacterial colonies become bright compared to the background [2].

It has been proved that, it is not necessary to use costly hardware and imaging system to collect the images of bacterial colonies. With the development of digital cameras and document scanners, images of bacterial colonies can be obtained easily [3]. Counting colonies of clonogenic assays using densitometric software has been introduced. This software is named as Clono-Counter (CC). One must have some experience to select the right parameters for CC [4].

Commercial colony counters available in the market has been reviewed and classified into two categories- First kind of counters which are not real automatic counters, they still require technicians to use probe or pen to touch each colony and register the count. Second type of counter is semi automatic or automatic counter, these high priced devices come with their own hardware. Digital cameras are now available at affordable prices. Efforts have been made to develop a system which can accept images obtained from these affordable devices. But the proposed counter has failed to count colonies grown over transparent medium because of low contrast and also due to existence of noises on the plate which makes difficult to differentiate between noises and colonies [5]. This problem is solved by using a method which deals with chromatic and achromatic images differently. This is achieved by examining the standard deviation of mean values from each colour channel R, G and B [6].

Along with colony enumeration, bacterial colony classification has also been considered. Colony classification here means counting colonies of a specific strain in a sample. This is obtained by using Support Vector Machine (SVM) classifier [7]. In case of heterotrophic bacteria counting, it has been revealed that colony counting based on image processing method is feasible. Not only it has the advantages of fast, simple and accurate, but also the counting result does not be affected by using inoculation, variety, shape and size of the colony. This method is also widely applicable for counting other type of microbial [8].

An algorithm based on scattering patterns of bacteria colonies which exhibit some specific features, which may be exploited for identification of various bacteria species, was proposed. Any defect of medium, possible structural and optical non-homogeneities may affect the analysis. Proposed approach was considered the case of bacteria colonies with the same shape, therefore the analysis of samples containing bacteria colonies with different shapes by proposed method can lead to significant errors. It should be pointed out as well, that all bacteria sample images should be recorded in the same illumination conditions [9].

A new automatic colony counting system, which makes use of image-processing technology to feasibly count white bacterial colonies in clear plates according to the RGB color theory, has been developed. Use of such image processing methods on white colony counting is feasible, which is not affected by colony morphology, size and inoculation method, but the influence of the irradiation light and the background color of the collected image is very serious. Therefore, use of closed source system to achieve lighting to avoid the impact of natural light is recommended [10].

Colony counting can be affected by numerous parameters related to the physical properties of the colony: size, shape, contrast, and overlapping colonies. Determinations of overlapping colonies have been deduced by shape analysis [11], distance transform and watershed algorithm is also proposed to segment overlapped colonies [8].

The purpose of this work is to develop a methodology to count bacterial colonies automatically using digital image processing. The proposed method will be easy to use, as it does not require any initial parameters; there is no need of any kind of manual intervention and experienced hands. Background artefacts are handled by using filter. It is always found easy to count colonies in a high contrast images, but this methodology gives equally acceptable results for low contrast images.

This paper is organized as follows: Introduction to Microorganisms is presented in Section 2. Methods for measurement of cell numbers are given in Section 3. The proposed methodology is described in Section 4. Results are presented in Section 5 and Section 6 consists of conclusion & discussion.

## 2. MICROORGANISMS

The microbial world is made up of micro-organisms and viruses. Viruses are non-cellular entities and cannot be considered microorganisms. Microorganisms are unicellular organisms (capable of existence as single cells), too small to be seen with the naked eye. There are five major groups of microorganisms, Archaea, Bacteria, Algae, Protozoa, and Fungi. Biologists recognize the existence of two fundamentally different types of cells in the microbial world, called procaryotic and eucaryotic cells. Eucaryotic cells have a "true" nucleus (the region of the cell that contains genetic information or DNA) because it is enclosed in a nuclear membrane; procaryotic cells are said to have a "primitive" nucleus because their DNA is not enclosed within a nuclear membrane. Archaea and Bacteria are procaryotic cells. Unicellular algae and protozoa and fungi are eucaryotic cells, similar to plants and animals. The nuclear region of a procaryotic cell is sometimes referred to as a nucleoid, but never as a nucleus [12].

Bacteria are unicellular procaryotic organisms. They are typically a few micrometres long. Bacteria come in wide variety of shapes and sizes called the morphology of the organism. The most common shapes are rod like and spherical. Unlike in multicellular organisms, increases in cell size (cell growth and reproduction by cell division) are tightly linked in unicellular organisms. Bacteria grow to a fixed size and then reproduce through binary fission, a form of asexual reproduction. Under optimal conditions, bacteria can grow and divide extremely rapidly, and bacterial populations can double as quickly as every 9.8 minutes. In cell division, two identical clone daughter cells are produced. To study more about growth of bacteria refer to [13].

## 3. MEASURING TECHNIQUES

Measuring techniques based on cell counting, involve direct counts, visually or instrumentally, and indirect viable cell counts.

*3.1 Direct microscopic counts* are possible using special slides known as counting chambers. Dead cells cannot be distinguished from living ones.

*3.2 Electronic counting* chambers count numbers and measure size distribution of cells. For cells the size of bacteria the suspending medium must be very clean. Such electronic devices are more often used to count eucaryotic cells such as blood cells.

*3.3 Indirect viable cell counts*, also called plate counts, involve plating out (spreading) a sample of a culture on a nutrient agar surface. The sample or cell suspension can be diluted in nontoxic diluents (e.g. water or saline) before plating. If plated on a suitable medium, each viable unit grows and forms a colony. Each colony that can be counted is called a colony forming unit (CFU) and the number of CFU's is related to the viable number of bacteria in the sample [14].

Proposed Methodology (PM) for counting colonies comes under indirect viable counts method, for which bacteria are cultured on a solid surface using some nutrients, under suitable environment conditions, so that they form colonies. Bacterial colony is a group of bacteria derived from single bacterium, by counting these colonies we can obtain cell count.

## 4. PROPOSED METHODOLOGY (PM)

### 4.1 Image Acquisition

First step towards the colony counting is to acquire the bacterial colony images. With the advancement in digital devices now days it becomes very easy to acquire a digital image of bacterial colonies cultured on a filter membrane. Images can be obtained with scanner or a digital camera. Although digital cameras capture images from the top, document scanners image the membrane from the bottom and therefore are more susceptible to errors caused by scratches at the bottom. Typically, scratches appear lighter in the image and are unlikely to be mistaken as colonies. Rarely, a line scratch crossing over a single colony may be counted as double.

**Fig 1: Bacterial Colonies**

To obtain high contrast colony images 2, 3, 5-triphenyletetrazolium chloride (TTC), a dye indicative of cellular metabolism that stains the bacterial colonies deep red with no coloration of the background surface, was added. With the use of dye bacterial colonies appear as bright red spots (as shown in Fig 1), which make counting process easy. It has been observed that TTC concentration has no effect on the background intensity. TTC concentrations below 12.5µg/ml produced insufficient contrast for automated counting. Contrast further improves until 50µg/ml TTC at which point more dye does not improve the image. It is concluded that a TTC concentration of 25µg/ml is sufficient to produce an image with high contrast between the colonies and the background [1].

### 4.2 Grayscale Image

Mathematically we can represent the colour image by mean of using 3 dimensional matrixes, one dimension for red colour and the second for the blue colour and the third one for the green colour. So we can take the colour image as a 3 matrices one for each colour. An RGB image sometimes referred to as a "true colour" image and it can be stored as an m-by-n-by-3 data array that defines red, green and blue colour components for each individual pixel. The colour of each pixel is determined by the combination of the red, green and blue intensities stored in each colour plane at the pixel's location. Graphics file formats store RGB images as 24 bit images,

where the red, green and blue components are 8 bits each. This yields a potential of 16 million colours. The precision with which a real-life image can be replicated has led to the nickname "true colour image". Moreover, 85% of the noise tends to be in "Intensity" component. So it is better to process "I" for noise-removal rather than the three R = G= B components [15].

A grayscale digital image is an image in which the value of each pixel is a single sample, that is, it carries only intensity information but no chromatic information. They are composed exclusively of shades of gray varying from black as the weakest intensity to white as the strongest. In addition to black and white, they consist of varying shades of gray.

After grayscale conversion Bacterial Colony image will look like as shown in Fig 2.

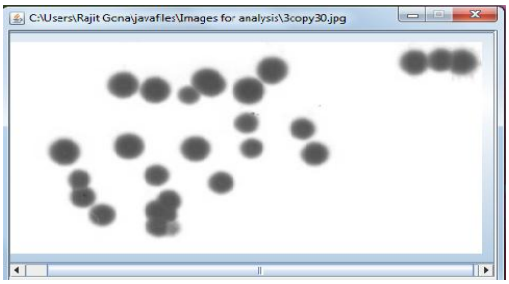


**Fig 2: BC image after grayscale conversion**

Essentially, standard grayscale images are actually eight bit black-and-white images such that for each pixel, there is a set of $2^8$ permissible values. Due to this, grayscale images have many shades of gray in between. The reason for differentiating these images from any other sort of colour images is that less information needs to be provided for each pixel. Gray colour is one in which all the red, green and blue components have equal intensities. So, it is necessary to specify only a single intensity value for each pixel.

To convert any colour to a grayscale representation of its luminance, first one must obtain the values of its red, green, and blue (RGB) primaries. Then, a certain percentage of the RGB values are added together. The weights depend on the exact choice of the RGB primaries, but are never fixed. Regardless of the scale employed (0.0 to 1.0, 0 to 255, 0% to 100%, etc.), the resultant number is the desired linear luminance value. There are three basic strategies to convert a colour image to a grayscale image; namely the lightness method, average method and luminosity method. Let us assume that R, G and B are the value of a pixel's Red, Green and Blue components respectively. Also let GY denote the resulting gray level for that pixel. The lightness method averages the most prominent and the least prominent colours.

$GY = (\max(R, G, B) + \min(R, G, B))/2$

The average method simply averages the values. So,

$GY = (R + G + B)/3$

The luminosity method is more sophisticated approach. It also averages the values but it forms a weighted average to account for human perception. Human eyes are more sensitive to green than other colours, so green is weighted most heavily in this approach. Colours in an image can be converted to a shade of gray by calculating the effective brightness or luminance of the colour and then using this value to create a shade of gray that matches the desired brightness. So,

$GY = 0.56\,G + 0.33\,R + 0.11\,B$

The lightness method generally tends to reduce the contrast of the resulting image. But for some images, the three algorithms produce very similar results [16].

## 4.3 Noise Removal

Elimination of noise is one of the major tasks to be done in image processing, as noise leads to the error in the image. Presence of noise is manifested by undesirable information, which is not at all related to the image under study, but in turn disturbs the information present in the image. It is translated into values, which are getting added or subtracted to the true gray level values on a gray level pixel. Noise is of many types. Thus image noise can be Gaussian, Uniform or impulsive distribution.

A digital filter is used to remove noise from the degraded image. Thus filter is an important subsystem of any signal processing systems. Thus filters are used for image enhancement, as it removes undesirable signal components from the signal of interest.

Noise reductions are basically classified into two types 1) linear techniques and 2) Non linear techniques. In linear techniques noise reduction formula is applied for all pixels of image linearly without classifying pixel into noisy and non noisy pixels. Drawback of linear algorithms is it damages the non noisy pixels because algorithm is applied for both noisy and non noisy pixels. Examples for linear filters are average, mean, median filters etc. Non linear Noise reduction is a two step process 1) noise detection and 2) noise replacement. In first step, location of noise is detected and in second step, detected noisy pixels are replaced by estimated value [17].

Although median filter is a useful image smoothing and enhancement technique, it also has some disadvantages. The median filter removes both the noise and the fine detail since it can't tell the difference between the two. Anything relatively small in size compared to the size of the neighborhood will have minimal affect on the value of the median, and will be filtered out. In other words, the median filter can't distinguish fine detail from noise. Traditional median filter and mean filter are used to reduce salt-pepper noise and Gaussian noise respectively. When these two noises exist in the image at the same time, use of only one filter method cannot achieve the desired result.

Therefore the Adaptive Median Filtering (AMF) has been applied widely as an advanced method compared with standard median filtering. The AMF performs spatial processing to determine which pixels in an image have been affected by impulse noise. The AMF classifies pixels as noise by comparing each pixel in the image to its surrounding neighbor pixels. The size of the neighborhood is adjustable, as well as the threshold for the comparison. A pixel that is different from a majority of its neighbors, as well as being not structurally aligned with those pixels to which it is similar, is labeled as impulse noise. These noise pixels are then replaced by the median pixel value of the pixels in the neighborhood that have passed the noise labeling test.

AMF is designed to eliminate the problems faced by the Standard Median Filter. AMF changes its behaviour based on the statistical characteristics of the image inside the filter window. Adaptive filter performance is usually superior to non-adaptive counterparts [18].

## 4.4 Object and Background Separation

The gray levels of pixels belonging to the object are substantially different from the gray levels of the pixels belonging to the background. Thresholding then becomes a simple but effective tool to separate objects from the background. The output of the thresholding operation is a binary image in which 0 (black) will indicate a pixel belonging to an object and 1 (white) will indicate the background. As binary images are easy to operate, other

storage format images are often converted into binary images when they are used for enhancement or edge detection [19].
Because of its efficiency in performance and its simplicity in theory, thresholding techniques have been studied extensively and a large number of thresholding methods have been published. Usually, automatic thresholding approaches are classified into two main groups: global and local. In global methods, a fixed threshold is used for the hole image, whereas in local methods the threshold changes dynamically (local methods are often used when the background is uneven due to the poor illumination condition) and the threshold value is computed for each pixel on the basis of information contained in a local neighbourhood of the pixel. In the context of global thresholding many algorithms have been reported in the literature. In bi-level thresholding, the histogram of the image is usually assumed to have one valley between two peaks, the peaks representing background and objects, respectively. There are two main approaches to the problem of locating the intensity threshold that ideally represents the bottom of this sometimes elusive histogram valley: parametric and non-parametric techniques. In the non-parametric case, we separate the two gray level classes in an optimum manner according to some posterior criterion, without estimating the parameters of the two distributions. The non parametric methods are more robust, and usually faster than the parametric [20].
In digital images, uniformity and shape of the objects play great roles in separating objects from the background. The amount of agreement of these two aspects of every binary image with the real image has been evaluated and results show that the performance of non parametric moment preserving thresholding method (Tsai 1985) is good as compared to others and can be easily extended to multilevel thresholding [21].
After applying filter and converting it into binary image the bacterial colonies will look like as shown in Fig 3.

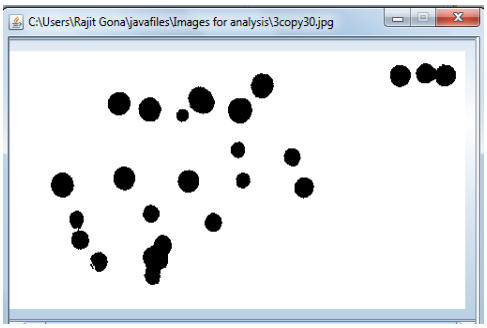


**Fig 3: Binary image of Bacterial Colonies**

## 4.5 Boundary Extraction

Next step towards colony counting is to obtain boundaries of colonies, which can be achieved by using morphological operations. Mathematical morphology is a well-founded non-linear theory of image processing. Its geometry-oriented nature provides an efficient framework for analysing object shape characteristics such as size and connectivity, which are not easily accessed by linear approaches. Morphological operations take into consideration the geometrical shape of the image objects to be analysed. Mathematical morphology is theoretically founded on set theory. It contributes a wide range of operators to image processing, based on a few simple mathematical concepts. The operators are particularly useful for the analysis of binary images, boundary detection, noise removal, image enhancement, and image segmentation.

An image can be represented by a set of pixels. A morphological operation uses two sets of pixels, i.e., two images: the original data image to be analysed and a structuring element. Basic operation of a morphology-based approach is the translation of a structuring element over the image and the erosion and/or dilation of the image content based on the shape of the structuring element. Morphological operations analyse and manipulate the structure of an image by marking the locations where the structuring element fits. In mathematical morphology, neighbourhoods are, therefore, defined by the structuring element, i.e., the shape of the structuring element determines the shape of the neighbourhood in the image.

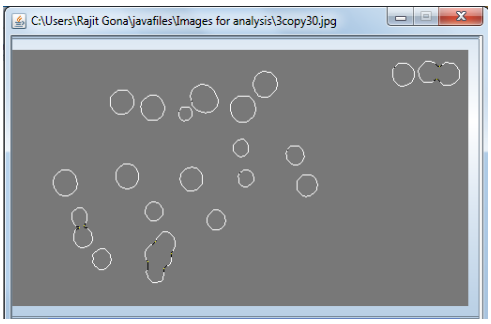


**Fig 4: Image showing boundaries of colonies**

The dilation is an expansion operator that enlarges binary objects. The erosion is a thinning operator that shrinks objects. Erosion and dilation can be combined to solve specific filtering tasks. Widely used combinations are opening, closing, and boundary detection. Morphological operations are very effective for detection of boundaries in a binary image [22] [23]. After achieving boundaries of colonies, we can count the colonies by traversing the boundaries as shown in Fig 4.

## 4.6 Counting Overlapped Colonies

Contour curvature features are one of the most important information that can be used for object recognition from shape. The points of maximum convexity, concavity and inflexion partition the image curves into relative stable segments which then can be matched against each other. Sometime these points are also called critical or salient points. Curvature, defined as the change rate of the slope, has been widely employed in different applications such as shape representation, feature extraction, corner detection and object recognition. The points where bacterial colonies overlap can be identified as high curvature point over the boundaries of colonies, or we can say a corner point. Therefore, a corner is defined as a "high-curvature point" on a simple digital arc or curve.
Overlapped colonies can be determined by finding high curvature points on a digital curve. Corner detection plays a critical role in image processing and pattern recognition in which many different approaches having been developed. These approaches can be broadly classified into two major categories: gray-level and boundary-based approaches. Gray-level approaches match corners using gray-level corner templates or compute the gradient at edge points while boundary-based approaches analyze the properties of boundary pixels to identify corners. Boundary-based corner detection method using *K*-cosine has been proposed. This method aims to attain robust corner detection for objects containing various corners [24] [25].

## 5. RESULTS

Proposed Methodology (PM) for Bacterial Colony (BC) counting has been tested for different type of filter images. The sample images have been taken from a unit involved in membrane technologies, where bacteria's counting is required to meet the quality standards of their products. Results obtained from PM have been compared with the manual count as shown in the Table 1.

**Table 1. Difference between manual & PM count**

| BC Image type | Manual count | Count by PM | Difference |
|---|---|---|---|
| High contrast, without overlapped colonies | 10 | 10 | 0 |
| High contrast, overlapped colonies | 93 | 91 | -2 |
| Low contrast | 30 | 29 | -1 |
| Low contrast, noisy | 39 | 37 | -2 |
| High contrast, more than four overlapping | 120 | 123 | +3 |
| High density | 203 | 201 | -2 |
| High density, noisy | 124 | 122 | -2 |

## 6. CONCLUSION AND DISCUSSION

Whether it is manual counting or automatic counting, use of different coloured dyes and easily available digital picture capturing and storing devices has made the bacterial counting task easy. Objective of this work is to propose a simple methodology with which colony counting task can be easily performed without using any initial training data.

Performance of proposed counter has been tested on different type of Bacterial Colony images; filter images having small no. of colonies, high density colony images, low contrast images, images with background noise. The results obtained with proposed counter have been compared with manual count. For high contrast images proposed counting methodology performed very well. Colony count of images containing dense colonies and images with low contrast was also within the tolerance limit of error.

It has been revealed that colony counting based on image processing method is feasible. Furthermore, the image processing method is also widely applicable for counting other kind of microbial and it greatly improves the accuracy and efficiency. Present work can be improved further for more complicated images as there is always a scope of improvement in digital image processing techniques.

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