Different binarization processes validated against manual counts of fluorescent bacterial cells

Tamminga, Gerrit G.; Paulitsch-Fuchs, Astrid H.; Jansen, Gijsbert J.; Euverink, Gert-Jan W.

Published in:
Journal of microbiological methods

DOI:
10.1016/j.mimet.2016.07.003

Publication date:
2016

Journal of Microbiological Methods 128 (2016) 118–124

Contents lists available at ScienceDirect

Journal of Microbiological Methods

journal homepage: www.elsevier.com/locate/jmicmeth

Different binarization processes validated against manual counts of fluorescent bacterial cells

Gerrit G. Tamminga [a,b,*], Astrid H. Paulitsch-Fuchs [a,c], Gijsbert J. Jansen [b], Gert-Jan W. Euverink [d]

[a] Wetsus, European centre of excellence for sustainable water technology, Leeuwarden, Netherlands
[b] Biotrack, Leeuwarden, Netherlands
[c] Medical University of Graz, Institute of Hygiene, Microbiology and Environmental Medicine, Graz, Austria
[d] Faculty of mathematics and natural sciences, products and processes for biotechnology, University of Groningen, Groningen, Netherlands

ARTICLE INFO

Article history:
Received 6 June 2016
Received in revised form 1 July 2016
Accepted 1 July 2016
Available online 2 July 2016

Keywords:
Microscopy
Automated-cell-count
Binarization
Fluorescence
Algorithm

ABSTRACT

State of the art software methods (such as fixed value approaches or statistical approaches) to create a binary image of fluorescent bacterial cells are not as accurate and precise as they should be for counting bacteria and measuring their area. To overcome these bottlenecks, we introduce biological significance to obtain a binary image from a greyscale microscopic image. Using our biological significance approach we are able to automatically count about the same number of cells as an individual researcher would do by manual/visual counting. Using the fixed value or statistical approach to obtain a binary image leads to about 20% less cells in automatic counting. In our procedure we included the area measurements of the bacterial cells to determine the right parameters for background subtraction and threshold values. In an iterative process the threshold and background subtraction values were incremented until the number of particles smaller than a typical bacterial cell is less than the number of bacterial cells with a certain area. This research also shows that every image has a specific threshold with respect to the optical system, magnification and staining procedure as well as the exposure time. The biological significance approach shows that automatic counting can be performed with the same accuracy, precision and reproducibility as manual counting. The same approach can be used to count bacterial cells using different optical systems (Leica, Olympus and Navitar), magnification factors (200× and 400×), staining procedures (DNA (Propidium Iodide) and RNA (FISH)) and substrates (polycarbonate filter or glass).

1. Introduction

Fluorescence microscopy is a generally accepted method in microbiology that can be used to detect e.g., cells, proteins, DNA, glycogen, polyhydroxybutyric acids with a variety of different staining procedures (e.g., Fluorescence in situ Hybridisation (FISH), staining of DNA) and fluorescent labels (e.g., propidium iodide, Acridine orange, Cy3, FITC). After proper evaluation of microscopic images by eye or by using software tools e.g., the total number of bacterial cells, cell numbers of specific bacterial species, ratio of viable and non-viable bacteria and bacterial cell area can be obtained from the data. However, straightforward comparisons between results obtained from different experiments are difficult. In an effort to standardize results, digital images of fluorescent labelled organisms or structures can be processed by (semi-)automated image analysis software packages (Grivet et al., 1999; O'Mahony et al., 2005; Selinummi et al., 2005; Thiel and Blaut, 2005). In this automatization process a grayscale image is converted into a binary (two possible pixel values, normally black and white) image. This conversion process is called thresholding. However, the application of an automated thresholding algorithm to discriminate the background (white; value = 0) from the objects of interest (black; value = 1) is challenging. Therefore, the use of a fixed threshold is accepted in current software approaches (O'Mahony et al., 2005). Some authors report the use of an empirically obtained fixed threshold based on their own reference dataset (Schönholzer et al., 1999; Thiel and Blaut, 2005). Nevertheless, even with an optically perfect system, a simple segmentation at a fixed threshold is not appropriate for a reliable and reproducible size measurement or to count different objects (Bjørnsen, 1986). For example, if a technician has the responsibility to manually set the threshold, the variability in the threshold is about 60%. If there are more technicians involved the variability drops to 40% (Webb et al., 2003). To overcome this bias, which is a critical step in automated image analysis, alternative approaches in the development of an automated flexible thresholding procedure are necessary (Mesquita et al., 2013).

Several attempts in that direction use pre-treatment(s) of the images (e.g., increasing contrast, subtract background algorithm or out of focus image, changing the histogram, sharpening, delineation) (Bloem et al., 1995; Klauth et al., 2004; Pernthaler et al., 2003). However, implementation of these steps can lead to significant loss of information. Compared to the results of a manual count, the outcome of the

* Corresponding author at: Wetsus, European centre of excellence for sustainable water technology, Leeuwarden, Netherlands.
E-mail address: g.tamminga@biotrack.nl (G.G. Tamminga).

http://dx.doi.org/10.1016/j.mimet.2016.07.003
0167-7012/

algorithms for the number of cells was lower and less accurate (coefficient of variation was 30% versus 10% respectively) (Bloem et al., 1995; Seo et al., 2010).

There are other approaches to objectively determine the threshold; all of them are based on statistical analyses of the histogram or intensity value of each individual pixel or/and their neighbours. The following six groups of threshold methods are accepted: histogram shape-based, clustering-based, entropy-based, attribute similarity based, spatial based and local based (Sezgin and Sankur, 2004).

An interesting method is the topological stable-state threshold method from the attribute similarity based class. In this method, groups of objects with identical intensity-values are predefined and correlated to an incremental increasing threshold level. Objects regarded as "noise", rapidly disappear when shifting the threshold (Pikaz and Averbuch, 1996). Another comparable algorithm is the stable count method. In this method each threshold will be applied (increasing the threshold by 1 up till the maximum). For each set of threshold a count of objects will be performed; if the count of objects remains constant the threshold is found (Russell et al., 2009).

Nonetheless, those algorithms make no use of pre-set parameters holding biological significance (e.g., area or/and size of a typical bacterial cell). This lack of implementation of biological key figures (to the best of our knowledge) in the current types of threshold algorithms leads us to our hypothesis: When implementing biological significant variables into a threshold algorithm a reliable, accurate, precise and reproducible estimation of area and counts of bacterial cells can be achieved.

Our study was designed broadly to test our hypothesis. We used different staining procedures (e.g., DNA and RNA staining protocols) on diverse surfaces (e.g., glass and polycarbonate). The samples were examined with four optical systems (OS) and images were taken at different magnification factors (e.g., 200× and 400×). The images were analysed manually and by using three different approaches of thresholding: i) a set value approach (Schönholzer et al., 1999; Thiel and Blaut, 2005), ii) a statistical approach (Otsu, 1979), and iii) an approach integrating biological significant variables.

This study presents an algorithm implementing biological significant variables that was especially developed to analyse images containing fluorescent labelled bacteria.

None of the existing methods (to the best of our knowledge) uses key numbers of the object of interest (OOI) to find the precise cut-off value to produce a binary image. We suggest implementing previously known data of the OOI's (in this case we assume an average area of a bacterial cell of 1 μm^2 (Palumbo et al., 1984; Srivastava and Srivastava, 2003)) into the concept of Pikaz and Russell (Pikaz and Averbuch, 1996; Russell et al., 2009) to perform a precise cut-off.

For every shift of the threshold, a count will be performed for the noise (smaller than the average area of a bacterium) and for the OOI (equal and larger than the average area of a bacterium). The threshold is found when the count of the noise is smaller than the count of the OOI. The main aim was to overcome the existing reduced sensitivity and accuracy using software algorithms to count bacteria compared to the results of manual counting of bacteria in microscopic images. The presented algorithm is dynamic, automated and applicable for at least four different optical systems.

2. Materials and methods

2.1. Bacterial strains and growth conditions

For all experiments F1 generations of the bacterial strains *Pectobacterium carotovorum* subsp. *carotovorum* (DSMZ 30169), *Escherichia coli* (DSMZ 301), *Aeromonas hydrophila* (DSMZ 30017) and *Enterococcus faecalis* (DSMZ 2570) were used. Bacteria were cultivated from the Cryo-bank in 5 mL TSB (Oxoid, CM0129) for 20 h. 100 μL of this culture was subcultured in 5 mL TSB for 16–24 h before taking a sample for DNA and RNA staining. DSMZ 30169 was grown at 20 °C, DSMZ 30017 at 30 °C and DSMZ 301 and DSMZ 2570 at 37 °C.

2.2. Staining protocols

2.2.1. DNA and RNA staining on microscopic glass slides

2.2.1.1. Sample preparation. Fixation of the bacteria cells: 10% F1 generation, 10% formaldehyde (37% *v*/*v*) and 80% sodium chloride solution (0.9% (*w*/*v*) NaCl) (*v*/*v*) were mixed well and incubated for 30 min at room temperature. After fixation, the bacteria were spotted on glass (diagnostic slides, 8 wells/6 mm) and air dried for 30 min. Dehydration (96% (*v*/*v*) ethanol) was applied to ensure total water removal and to allow easy transport of the staining dye into the bacteria cells.

2.2.1.2. DNA staining. The staining solution was added and samples were incubated for 10 min in the dark at room temperature. Staining solution A was of 0.03 mg/mL propidium iodide (Fluka, Sigma-Aldrich ecno. 2470810) dissolved in Milli Q water. Staining solution B was of 0.03 mg/mL propidium iodide dissolved in 0.1 M sodium phosphate buffer pH 7.0 (75.6 mL 0.1 M Na_2HPO_4 and 24.4 mL 0.1 M NaH_2PO_4). The slides were washed with Milli Q (staining solution A) or with SCC (staining solution B) (SCC 1×: 0.3 M NaCl, 15 mM sodium citrate, pH 7.0) for 15 min in the dark at room temperature.

2.2.1.3. RNA staining. The RNA staining solution was added to the fixed air dried and dehydrated bacterial cells spotted on glass slides (see above). Samples were incubated for 60 min in the dark at 45 °C. The staining solution was of probe EUB338 (Amann et al., 1990) (12 ng/μL) (sequence; 5′-GCT GCC TCC CGT AGG AGT-3′), hybridisation buffer (0.9 M NaCl, 20 mM Tris-HCl pH 7.5, 0.01% (*w*/*v*) SDS and 20% (*v*/*v*) formamide, 0.5% (*v*/*v*) Triton X-100). The slides were washed with washing buffer (0.9 M NaCl, 20 mM Tris-HCl pH 7.5 and 5 mM EDTA pH 8.0) for 15 min in the dark at room temperature.

2.2.1.4. Sample preservation. Slides were air dried in the dark and each spot was covered with 5 μL mounting medium (Invitrogen, I7224 component H) to prevent the degradation of the fluoro-chrome during the storage period (one week to several weeks, in the dark at room temperature).

2.2.2. DNA and RNA staining on polycarbonate filters

2.2.2.1. Sample preparation. The bacterial solution was diluted into various bacterial concentrations (between about $5 \cdot 10^3$ and about $5 \cdot 10^5$ per mL) and filtered through (1 or 10 mL sample) filters with a pore size of 0.4 μm (Millipore, HTBP01300). Fixation was performed by adding formalin solution (3.7% (*v*/*v*)) on to the filter for 30 min at room temperature. After removal of the formalin solution by pressure, dehydration fluid (96% (*v*/*v*) ethanol) was applied and removed by pressure.

2.2.2.2. DNA staining. Staining solution A or B (see DNA staining on microscopic glass slides) was applied for 10 min in the dark at room temperature. After staining the appropriate washing solutions for the different staining solutions were applied and removed by pressure. The filters were photographed directly.

2.2.2.3. RNA staining. Staining solution (see RNA staining on microscopic glass slides) was added (100 μL every 15 min (4 times); in the dark at 45 °C). After staining, the samples were washed three times with washing buffer. The filters were photographed directly.

Table 1
Optical systems.

OS	Microscope type	Lamp type	Camera type
1	Olympus (BH2)	Olympus mercury (BH2-RFL-T3)	Luminera, LM135
2	Olympus (BX43)	Lumen dynamics mercury XCITE (120Q)	Olympus, XM10
3	Leica (DM2500)	Leica mercury lamp (EL6000)	Leica, DFC450C
4	Navitar[a]	Navitar LED	Luminera, infinity III camera 3–1 M

[a] http://www.navitar.com (America).

2.3. Data acquisition

The four different optical systems (OS) that were used are summarized in Table 1.

The image conditions and pixel resolutions for the different optical systems are summarized in Table 2. Calibration of the OS was performed using a micro scale measuring staff (Carl Zeiss, Jena, Germany) with steps of 10 μm.

The images were taken with different exposure times varying from 90 to 1500 ms.

2.4. Data processing

We used three different approaches to process the images: i) a set value approach (SV). This approach uses an arbitrary value for obtaining a binary image. ii) A statistical approach (SA). This approach uses the calculation method of Otsu (Otsu, 1979) for producing a binary image. iii) An integration of biological significant variables approach (IBS). This approach uses the shift of threshold and counting of noise and OOI to determine a cut-off value when the count of OOI was larger than the count of the noise. At this point the threshold was set. The software used was ImageJ version 1.47 m (Abràmoff et al., 2004).

Before obtaining a binary image we set the minimum value to 0 (e.g., if the minimum value in the image was 20, then 20 was subtracted from all grey values of the image). After that we applied a subtract background method (Sternberg, 1983) (this method can handle zero values).

After obtaining a binary image the objects were counted based on area and intensity, using an overlay of the original and the binary image. Objects that were too small for a bacterium (calculated smallest rod (Palumbo et al., 1984) in pixels minus 1 pixel; calculations were based on calibrations per OS with a micro scale measuring staff) or had a lower intensity than the average background of the image were not counted. These two selection criteria were used for all three threshold approaches.

Table 2
Image conditions and pixel resolution.

OS	Magnification	Image resolution (pixels)	Storage format	Image type	File size (MB)	Pixel resolution (pixels/μm)
1	200×	1392 by 1040	TIFF	8 bit grayscale LUT[a]	1.4	4.0
1	400×	1392 by 1040	TIFF	8 bit grayscale LUT[a]	1.4	8.0
2	400×	1376 by 1038	BMP	32 bit RGB	5.4	6.3
3	400×	2560 by 1920	TIFF	8 bit grayscale LUT[a]	4.7	8.2
4	~200×	1392 by 1040	TIFF	8 bit grayscale LUT[a]	1.4	3.1

[a] Lookup tables for displaying each of the 256 possible pixel values.

Table 3
Average and standard deviation of the ratio software count versus manual count of the number of bacteria counted in each image using OS1, OS2, OS3 and OS4 at 200 and 400-fold magnification. SV: Set Value approach; SA: Statistical Approach; IBS: Integrating Biological Significant approach.

Optical system (n images)	Average [%] ± SD		
	SV	SA	IBS
OS1 400× (30)	70 ± 15	64 ± 17	94 ± 12
OS1 200× (10)	91 ± 8	97 ± 4	110 ± 13
OS2 400× (10)	84 ± 9	80 ± 10	100 ± 12
OS3 400× (10)	82 ± 19	80 ± 18	101 ± 11
OS4 ~200× (10)	92 ± 9	93 ± 8	106 ± 6
All (70)	80 ± 16	77 ± 19	100 ± 13

3. Results

The images were processed according to the description in material and methods. Each binarized image was used for automated and manual counting. For each OS the minimal size of the OOI was determined in pixels and was dependant on OS and magnification factor. The following values were obtained: OS1 200× <4; OS1 400× <15; OS2 400× <8; OS3 400× <15; OS4 200× <3 pixels. The original images were used for manual counting of the bacteria within ImageJ. Each bacterium was visually evaluated and counted. ImageJ marks already counted bacteria to prevent that single bacteria were counted multiple times.

For all approaches (3 automated methods and 1 manual) the data were recorded and summarized in Excel including the x,y coordinates of each bacterium in the images.

The total average of the ratio software/manual counts of all the processed images taken with OS1, OS2, OS3 and OS4 at 200 and 400-fold magnification are presented in Table 3. An average of 100% means that the automated counted the same number of bacteria as the manual method.

A normal distribution of the data sets for the different software approaches was confirmed using the Kolmogorov-Smirnov test ($\alpha = 0.05$). Therefore, a two tailed paired two-sample *t*-test was done comparing the results of the software bacterial counts to the manual bacterial counts set at 100% ($\alpha = 0.05$). The total number of bacteria counted with the IBS approach is equal to the total number of bacteria that were counted manually (ratio 100% ± 13, $p > 0.05$). The total number of bacteria that were counted with the SV approach (ratio 80% ± 16) and SA approach (ratio 77% ± 19) were lower than the total bacteria that were counted manually ($p < 0.05$).

Fig. 1 shows the thresholds for the grey values for all 70 images and for all three thresholding approaches. In the SV approach the same threshold for every image is used. The threshold values for the SA approach shows a broad variability whereas the dynamics of the IBS thresholding approach the threshold values stay within a certain band with.

We compared our IBS, SV, and SA approaches on images containing bacterial cells with high fluorescence intensity with images containing bacterial cells with low fluorescence intensity. Fig. 2 presents an image with objects with high fluorescence intensity (DNA staining) which were processed using the three different software approaches. According to visual inspection the IBS (D) approach shows that the selected area is very similar to the area of the bacteria in the original grey scale image (A). The SV (B) and SA (C) approaches overestimate the bacterial area compared to the bacterial area in the original image (A).

However, using an image with low fluorescence intensity bacteria the IBS approach behaves similar whereas the SV approach (B)

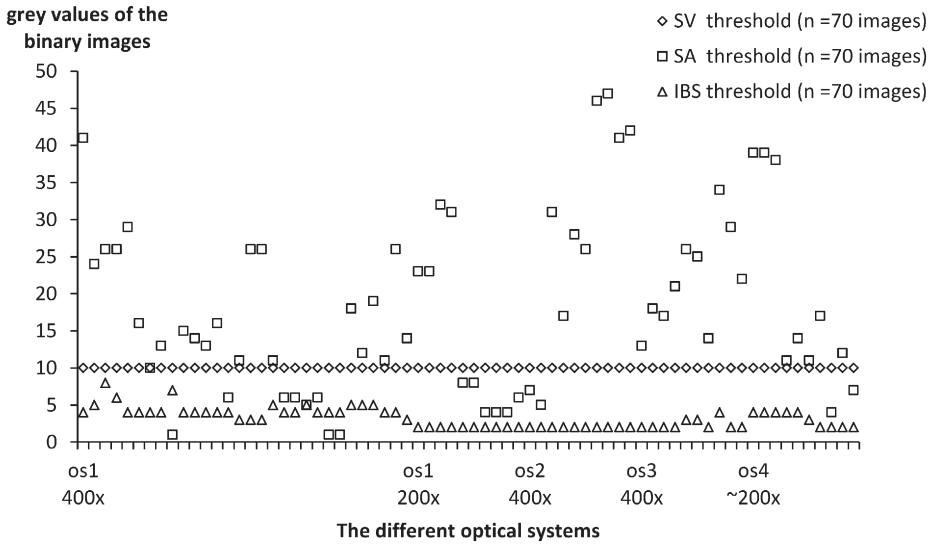

Fig. 1. Different grey values to obtain a binary image. SV: Set Value approach; SA: Statistical Approach; IBS: Integrating Biological Significant approach.

underestimates and SA approach (C) overestimates the bacterial area compared to the bacterial area in the original image (A) (Fig. 3).

In Table 4 the area averages and their coefficients of variation (CV) are presented for each tested OS as well as an overall summary of the different approaches with no respect to the OS.

While the IBS approach gives the values closest to the natural size of a bacterium, SA and SV approaches are more than twice as large as the natural size of a bacterium (~2 μm^2, largest rod (Palumbo et al., 1984)).

According to the results (Table 4, Figs. 2 and 3) the IBS approach (average size for a bacterial cell = 1.97 μm^2) displays the best match with the original bacterial area between 1–2 μm^2. For the SV and SA approaches the bacterial areas are determined to be 1.5 and 2 times larger, respectively. The fluctuation within the size evaluation of the bacterial cells is ~25% for the IBS approach whereas this fluctuation for SV and SA approach is ~56% and ~49%, respectively.

3.1. Validation of the counts based on coordinates of the objects (software/manual)

The approach as described above allows only conclusions about the total cell count numbers, positions of the bacteria within in the image (coordinates of the OOI) are not used for data validation. In the

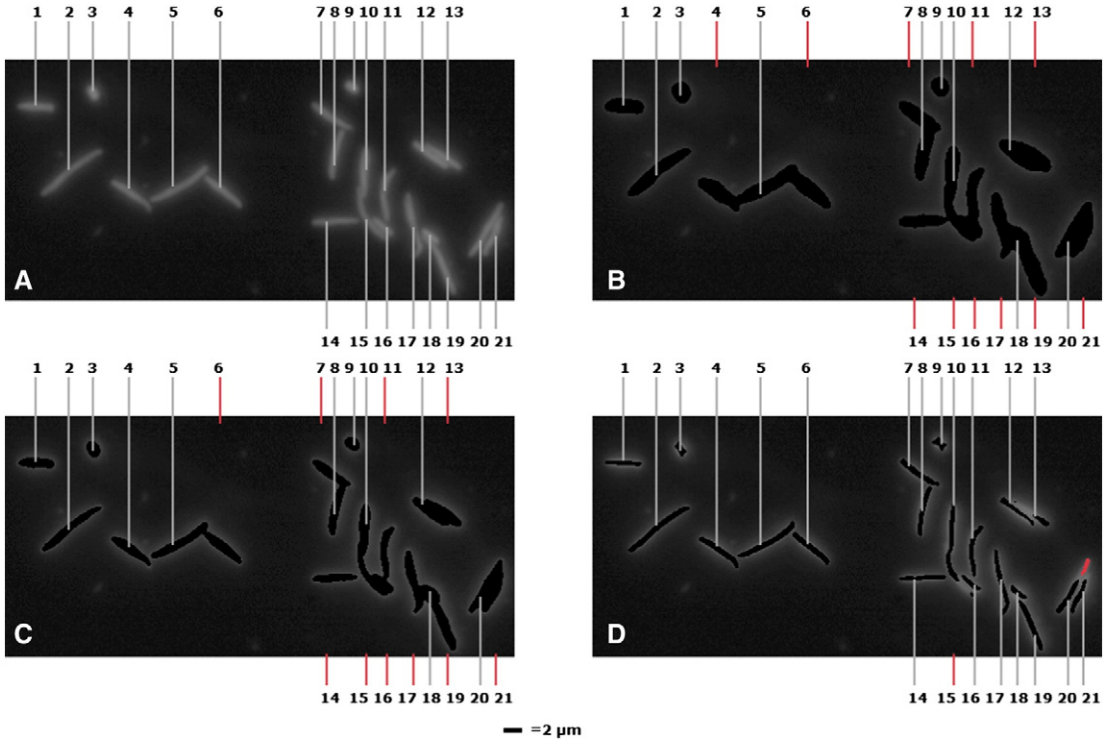

Fig. 2. Visual comparison of the three different software approaches; SV (B), SA (C) and IBS (D) with the original image (A). *Escherichia coli* (early growth stage) was stained with propidium iodide and imaged with OS2. Exposure time was 100 ms and the magnification factor 400×. Figures show the bacterial count in the original (A) (numbers 1 until 21) and compared with three software approaches: SV (10), SA (11) and for IBS (20). The red lines indicate missing counts for each approach. Notice that IBS has 1 extra object (shown in red). SV: Set Value approach; SA: Statistical Approach; IBS: Integrating Biological Significant approach.

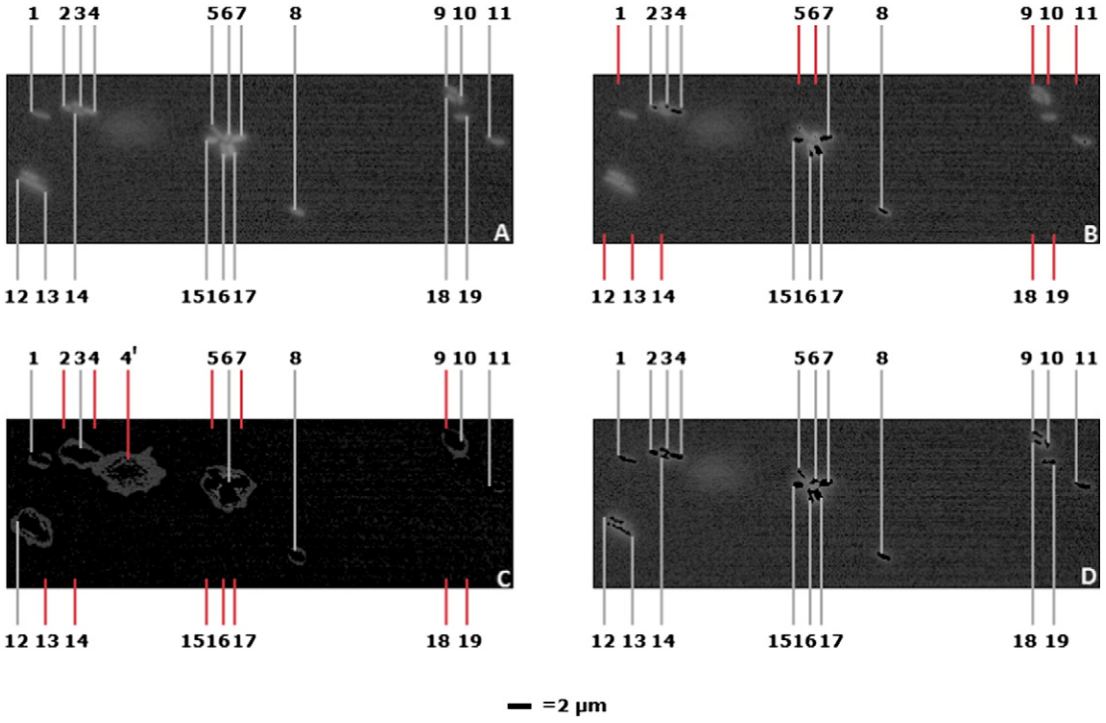

Fig. 3. Visual comparison of the three different software approaches; SV (B), SA (C) and IBS (D) with the original image (A). *Escherichia coli* was stained with probe EUB338 and imaged with OS2. Exposure time was 100 ms and the magnification factor 400×. Figures show the bacterial count in the original (A) (numbers 1 until 19) and compared with three software approaches: SV (8), SA (8) and for IBS (19). The red lines indicate missing counts for each approach. Notice that SA has 1 extra object (4′). SV: Set Value approach; SA: Statistical Approach; IBS: Integrating Biological Significant approach.

following paragraph we determine if the same bacteria are counted using the three different approaches and the manual counting by determining the X,Y coordinates of the objects that are counted.

Fig. 4 visualizes the importance of the position of the object in the data evaluation, by explaining the variance of the manual count (A and B are the extremes), the area of possible coordinates of the object (dotted lines) together with its orientation (which is unknown) and the possibilities of coordinates which relate to the one click for manual count.

This can be calculated with Eq. ((1) as follows:

$$\left(\left(\frac{pr^2}{swrb}\right)\times 2\right)-1 = sl^2, \qquad (1)$$

where pr is the pixel resolution [pipxels/µm], swrb is the smallest widh for a rod bacterium (assumed as 1/3 of a micrometre) [pipxels/µm] and sl^2 is the square of the total pixels around the manual coordinate.

According to pixel resolution and smallest width for a rod bacterium, the given Eq. ((1) was generated in this study to describe the area of total pixels around the manual coordinate of each object per OS and magnification factor. Table 5 summarizes the pixel resolution (pr), smallest width for a rod bacterium (swrb) and total pixels around the manual coordinate (sl^2) per OS.

All pictures where evaluated based on Eq. ((1) and the results are presented in Table 6, where the IBS approach clearly outperforms the SV and SA approach. A value of 100% means that all the bacteria counted by the software algorithm are the same bacteria that are counted by the manual method. The summarized data for all images and algorithms as

Table 4
Average bacterial area and their variation coefficients per OS as well as an overall summary of the different approaches with no respect to the OS.

Optical system	Magnification	N (images)/N (bacteria)	Approach	Average [µm^2]	Coefficients of variation [%]
1	200×	10/3529	SV	3.04	60.7
1	400×	30/4382		5.06	53.9
2	400×	10/676		4.73	34.9
3	400×	10/10,714		3.51	41.4
4	~200×	10/5226		2.46	44.2
1	200×	10/3766	SA	3.11	6.0
1	400×	30/4232		4.83	41.9
2	400×	10/673		3.53	15.1
3	400×	10/11,063		2.18	18.0
4	~200×	10/5279		2.00	15.1
1	200×	10/4292	IBS	2.32	33.8
1	400×	30/6148		1.85	18.0
2	400×	10/860		2.20	19.8
3	400×	10/12,917		1.60	14.9
4	~200×	10/5850		2.09	21.9
All	Varying	70/24,527	SV	4.13	56.4
All	Varying	70/25,012	SA	3.61	49.1
All	Varying	70/30,067	IBS	1.97	25.0

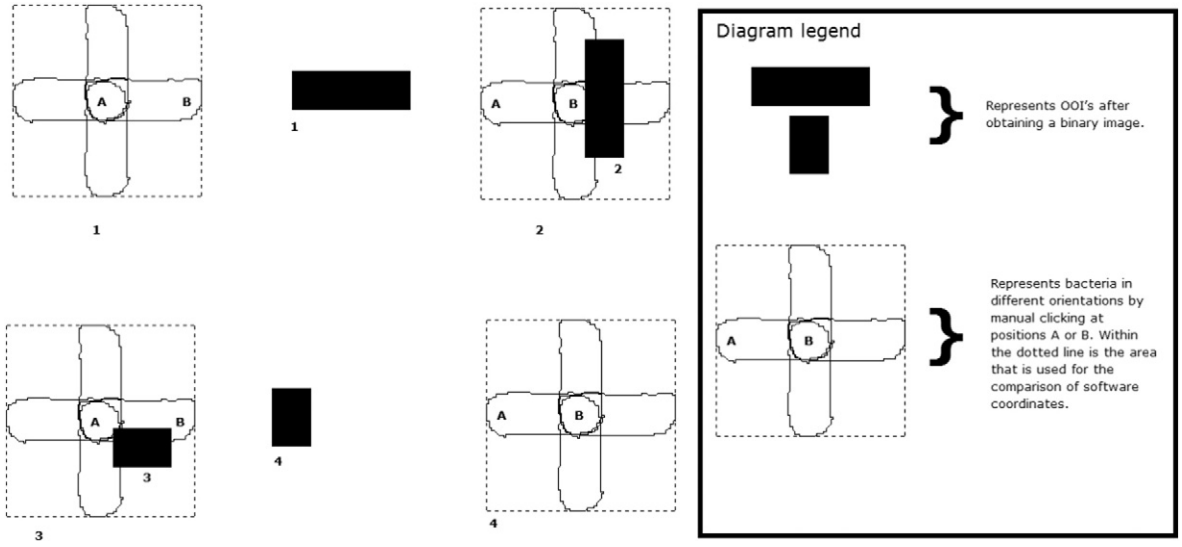

Fig. 4. Schematic drawing of an overlay of manual and software count. This example shows 4 possible situations. In situation 1 and 4 the OOI's are not laying within the dotted line. Therefore the software is failing to recognize the manual appointed bacteria. For situations 2 and 3 a recognition of the OOI is possible and therefore the software is working properly. As an example for the position of manually clicking the most extreme positions are presented (A and B). The dotted line is based on those extremes (see Eq. ((1)).

well as the detailed analyses per OS and magnification factors are given in Table 6.

The counts based on coordinate comparison of manual values and software values for the different software approaches are according to the Kolmogorov-Smirnov test ($\alpha = 0.05$) not normally distributed. Therefore, a chi-square test was performed comparing the results of the software counts to the manual bacterial counts set at 100% ($\alpha = 0.05$). The total number of bacteria counted with the IBS approach is equal to the total number of bacteria that were counted manually (ratio 98 ± 4, $p > 0.05$). The total amount of bacteria counted with the SV approach (ratio 93% ± 8) and SA approach (ratio 94% ± 11) were lower than the total amount of bacteria that were counted manually ($p < 0.05$).

4. Discussion

Since the acceptance of fluorescence microscopy for total bacterial count, specific bacterial count and area measurements numerous software methods were developed to decrease the processing time in order to achieve fast results (Grivet et al., 1999; O'Mahony et al., 2005; Selinummi et al., 2005; Thiel and Blaut, 2005). However, the difference between automated bacterial counts in microscopic images and the variation (30% CV) in the total bacterial counts and/or bacterial area measurements are still too high (Bloem et al., 1995; Seo et al., 2010).

In the automatization process a grayscale image is converted into a binary image (two possible pixel values, normally black and white). This converting process is called thresholding. The amount of counted objects is depended on the threshold value. A threshold value that is not properly chosen or calculated, results in an under- or overestimation of the total bacterial count in a microscopic image.

State of the art software methods either use a fixed value (SV) for the threshold which is empirically obtained from the data set (and therefore not generally applicable) (Schönholzer et al., 1999; Thiel and Blaut, 2005) or a statistical approach such as Otsu's method (SA) (Otsu, 1979).

Our study confirms the shortcomings of both widely used approaches (SA and SV) by showing that the ratio software/manual count is only ~80% with a CV of ~20%. In this study we introduce an addition to the threshold approach of Russell et al. (2009) by including biological important parameters as bacterial size and area (IBS). When this approach is used to count bacterial cells in a microscopic image we obtain a ratio software/manual count of ~100% with a CV of ~10%. Statistical analyses with the paired two-sample *t*-test ($\alpha = 0.05$; $p > 0.05$) confirm that the IBS approach results in total bacterial numbers that are equal to the total bacterial numbers that are counted manually. The SV and SA approaches result in different total bacterial numbers compared to a manual count.

When the SV or SA approach was used to convert a grey scale image into a binary image the mean area of the bacteria was ~4.1 ± 56% μm^2 and ~3.6 ± 49% μm^2, respectively. Compared to a largest rod of ~2 μm^2 (Palumbo et al., 1984) the values of both approaches overrate the area of a typical bacterial cell. The main reason for this is that these two approaches merge different bacterial cells into one large area as well as assigning larger areas to single bacterial cells (see also Fig. 2). We therefore conclude that a fixed threshold value does not yield reliable area measurements (Bjørnsen, 1986). SV and SA approaches on an image with a low contrast (e.g., bad illumination or low DNA/RNA content in the bacterial cell) underestimate the area of a bacterial cell. (see Fig. 3). The IBS approach yields an average area of ~2 ± 25% μm^2. We do notice that the average area with its CV is larger

Table 5
Summary of the pixel resolution (pr), smallest width for a rod bacterium (swrb) and total pixels around the manual coordinate (sl^2) in OS 1–4 with the different magnifications.

OS	pr [pixels/μm]	swrb [pixels/μm]	Total pixels around the manual coordinate
1 (200×)	4.0	1.33	0529
1 (400×)	8.0	2.67	2209
2 (400×)	6.3	2.10	1369
3 (400×)	8.2	2.73	2401
4 (~200×)	3.1	1.03	0289

Table 6
Average ratios (±standard deviation) based on coordinate comparison for all OS and magnification factors.

Optical system (n images)	Mean [%] ± SD		
	SV	SA	IBS
OS1 400× (30)	91 ± 10	92 ± 14	97 ± 6
OS1 200× (10)	93 ± 9	99 ± 1	99 ± 1
OS2 400× (10)	99 ± 2	98 ± 2	100 ± 0
OS3 400× (10)	97 ± 3	93 ± 16	100 ± 0
OS4 ~200× (10)	91 ± 10	91 ± 6	98 ± 3
All (70)	93 ± 8	94 ± 11	98 ± 4

(2.5 μm^2) than the largest rod ~2 μm^2 (Palumbo et al., 1984). Probably the culturing time has an effect on the size of the bacteria. We observed the presence of larger bacteria within the different experiments.

Additionally, we validated the algorithms by comparing the coordinates of the OOI's of the manual (gold standard) and software count. Although Eq. ((1) and our assumptions give a rather large possible area for the position of a cell in an image (2 à 3 times as big as the normal area of a bacterium, see Fig. 4), the accuracy of the data for the IBS approach is promising. The SV and SA approaches result in lower total bacterial counts than a manual approach. The IBS approach is statistically equal (chi-squared test, $\alpha = 0.05$; $p > 0.05$) to the manually achieved data set (Table 6). If we had outlined the bacteria manually and compared those coordinates with the software coordinates, the data could be even more accurate (presumably with >20%).

The IBS approach is not only limited to fluorescence microscopic images. For instance, we successfully analysed phase contrast and bright field images (data not shown) to calculate e.g., staining efficiencies. Also images that contained non-biological particles like electrospray droplets (Agostinho et al., 2012) could be automatically analysed using the IBS approach.

We were able to show that every image has a specific threshold with respect to the optical system, magnification and staining procedure as well as the exposure time (Fig. 1). As a result of this, the use of a uniform value (SV) for the creation of a binary image, for different optical systems and staining procedures is not reliable (O'Mahony et al., 2005). Even if the value is empirically estimated no accurate data can be obtained using the SV approach (Schönholzer et al., 1999; Thiel and Blaut, 2005). The SA approach involves the possibility of differences in threshold values with respect to the optical system, magnification and staining procedure like the IBS. Nevertheless, the exposure time has an effect within the SA approach: increased values of exposure times will affect the size of the OOI's and they will appear larger than they are in reality. If the image is not obtained with the right exposure time the outcome of the SA approach is that multiple objects are identified from originally single objects (Fig. 2). The IBS approach has a major advantage over the SV and SA approaches because this algorithm is only reliant on the pixel resolution and the obtained results are not affected by differences in exposure times (Table 4).

In this study all images were processed and no limitations were observed. However, the IBS approach is restricted to area selections for noise and OOI. At the time that OOI is larger than the selection criteria (e.g., clumps of bacteria (bacterial cells are packed to each other at high density)) these won't be detected as single bacterial cells as shown within in this study. The method has not been tested for counting filamentous organisms (e.g. fungi, actinomycetes) and might be limited in that respect as well.

In conclusion the results obtained with the IBS approach are equal to manual count and superior to the other two accepted approaches (SV and SA). The IBS approach has been proven to be reliable, accurate, precise and reproducible compared with the manual count regardless of the choice of optical systems (Leica, Olympus or Navitar), magnification factors (200× or 400×), staining procedures (DNA (Propidium Iodide) or RNA (FISH)) and substrates (polycarbonate filter or glass).

Acknowledgement

This work was performed in the TTIW-cooperation framework of Wetsus, centre of excellence for sustainable water technology (www.wetsus.nl). Wetsus is funded by the Dutch Ministry of Economic Affairs. The authors like to thank Jan Brouwer, Erwin Frijling and Antoine Engelaar for the fruitful discussions. We also thank the participants (Biotrack and University of Groningen) of the research theme "DNA detection technologies" for the fruitful discussions and their financial support.

References

Abràmoff, M.D., Magalhães, P.J., Ram, S.J., 2004. Image processing with ImageJ. Biophoton. Int. 11, 36–41. http://dx.doi.org/10.1117/1.3589100.

Agostinho, L.L.F., Tamminga, G.G., Yurteri, C.U., Brouwer, S.P., Fuchs, E.C., Marijnissen, J.C.M., 2012. Morphology of water electrosprays in the simple-jet mode. Phys. Rev. E Stat. Nonlinear Soft Matter Phys. 86.

Amann, R.I., Binder, B.J., Olson, R.J., Chisholm, S.W., Devereux, R., Stahl, D.A., 1990. Combination of 16S rRNA-targeted oligonucleotide probes with flow cytometry for analyzing mixed microbial populations. Appl. Environ. Microbiol. 56, 1919–1925. http://dx.doi.org/10.1111/j.1469-8137.2004.01066.x.

Bjørnsen, P.K., 1986. Automatic determination of bacterioplankton biomass by image analysis. Appl. Environ. Microbiol. 51, 1199–1204.

Bloem, J., Veninga, M., Shepherd, J., 1995. Fully automatic determination of soil bacterium numbers, cell volumes, and frequencies of dividing cells by confocal laser scanning microscopy and image analysis. Appl. Environ. Microbiol. 61, 926–936.

Grivet, M., Morrier, J.-J., Souchier, C., Barsotti, O., 1999. Automatic enumeration of adherent streptococci or *Actinomyces* on dental alloy by fluorescence image analysis. J. Microbiol. Methods 38, 33–42. http://dx.doi.org/10.1016/S0167-7012(99)00074-3.

Klauth, P., Wilhelm, R., Klumpp, E., Poschen, L., Groeneweg, J., 2004. Enumeration of soil bacteria with the green fluorescent nucleic acid dye Sytox green in the presence of soil particles. J. Microbiol. Methods 59, 189–198. http://dx.doi.org/10.1016/j.mimet.2004.07.004.

Mesquita, D.P., Amaral, A.L., Ferreira, E.C., 2013. Activated sludge characterization through microscopy: a review on quantitative image analysis and chemometric techniques. Anal. Chim. Acta 802, 14–28. http://dx.doi.org/10.1016/j.aca.2013.09.016.

O'Mahony, R., Basset, C., Holton, J., Vaira, D., Roitt, I., 2005. Comparison of image analysis software packages in the assessment of adhesion of microorganisms to mucosal epithelium using confocal laser scanning microscopy. J. Microbiol. Methods 61, 105–126. http://dx.doi.org/10.1016/j.mimet.2004.11.020.

Otsu, N., 1979. A threshold selection method from gray-level histograms. IEEE Trans. Syst. Man Cybern. 9, 62–66. http://dx.doi.org/10.1109/TSMC.1979.4310076.

Palumbo, A.V., Rublee, P.A., Miklas, J., 1984. Size of suspended bacterial cells and association of heterotrophic activity with size fractions of particles in estuarine and coastal. Appl. Environ. Microbiol. 48, 157–164.

Pernthaler, J., Pernthaler, A., Amann, R.I., 2003. Automated enumeration of groups of marine picoplankton after fluorescence in situ hybridization. Appl. Environ. Microbiol. 69, 2631–2637. http://dx.doi.org/10.1128/AEM.69.5.2631.

Pikaz, A., Averbuch, A., 1996. Digital image thresholding, based on topological stable-state. Pattern Recogn. 29, 829–843. http://dx.doi.org/10.1016/0031-3203(95)00126-3.

Russell, R.A., Adams, N.M., Stephens, D.A., Batty, E., Jensen, K., Freemont, P.S., 2009. Segmentation of fluorescence microscopy images for quantitative analysis of cell nuclear architecture. Biophys. J. 96, 3379–3389. http://dx.doi.org/10.1016/j.bpj.2008.12.3956.

Schönholzer, F., Hahn, D., Zeyer, J., 1999. Origins and fate of fungi and bacteria in the gut of *Lumbricus terrestris* L. studies by image analysis. FEMS Microbiol. Ecol. 28, 235–248.

Selinummi, J., Seppälä, J., Yli-Harja, O., Puhakka, J.A., 2005. Software for quantification of labeled bacteria from digital microscope images by automated image analysis. Biotechniques 39, 859–863. http://dx.doi.org/10.2144/000112018.

Seo, E.-Y., Ahn, T.-S., Zo, Y.-G., 2010. Agreement, precision, and accuracy of epifluorescence microscopy methods for enumeration of total bacterial numbers. Appl. Environ. Microbiol. 76, 1981–1991. http://dx.doi.org/10.1128/AEM.01724-09.

Sezgin, M., Sankur, B., 2004. Survey over image thresholding techniques and quantitative performance evaluation. J. Electron. Imaging 13, 146–165. http://dx.doi.org/10.1117/1.1631316.

Srivastava, S., Srivastava, P.S., 2003. Understanding Bacteria. first ed. Springer, Netherlands, Dordrecht http://dx.doi.org/10.1007/978-94-017-0129-7.

Sternberg, S.R., 1983. Biomedical image processing. Computer (Long Beach Calif.) 16, 22–34. http://dx.doi.org/10.1109/MC.1983.1654163.

Thiel, R., Blaut, M., 2005. An improved method for the automated enumeration of fluorescently labelled bacteria in human faeces. J. Microbiol. Methods 61, 369–379. http://dx.doi.org/10.1016/j.mimet.2004.12.014.

Webb, D., Hamilton, M.A., Harkin, G.J., Lawrence, S., Camper, A.K., Lewandowski, Z., 2003. Assessing technician effects when extracting quantities from microscope images. J. Microbiol. Methods 53, 97–106. http://dx.doi.org/10.1016/S0167-7012(02)00228-2.