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Abstract

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Automated quality assessment of autonomously acquired microscopic images of fluorescently stained bacteria

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Abstract:

Quality assessment of autonomously acquired microscopic images is an important issue in high-throughput imaging systems. For example, the presence of low quality images (≥ 10%) in a dataset significantly influences the counting precision of fluorescently stained bacterial cells. We present an approach based on an artificial neural network (ANN) to assess the quality of such images. Spatially invariant estimators were extracted as ANN input data from subdivided images by low level image processing. Different ANN designs were compared and > 400 ANNs were trained and tested on a set of 25000 manually classified images. The optimal ANN featured a correct identification rate of 94% (3% false positives, 3% false negatives) and could process about 10 images per second. We compared its performance with the image quality assessment by different humans and discuss the difficulties in assigning images to the correct quality class. The computer program and the documented source code (VB.NET) are provided under General Public Licence.
**Introduction:**

High throughput imaging has become a widely applied methodology in scientific research, e.g., for quantification of viral infection or phenotyping (1,2). In aquatic microbial ecology, motorized epifluorescence microscopes and image analysis systems are used to evaluate preparations of multiple fluorescently stained bacteria in a fully automated manner (3,4). Screening large numbers of samples greatly enhanced the scope of ecological studies and allows for larger spatial and temporal resolution in sampling (5-7). However, producing large amounts of image data raises new challenges in quality control, image processing and data evaluation (1).

Autonomous image acquisition from samples of environmental bacteria is prone to produce a certain amount of images that cannot be evaluated. Preparations subjected to autonomous imaging, often without prior manual inspection, may be of low quality for various reasons. Commonly encountered problems are fields of view (FOVs) with no cells, too high cell densities or an inhomogeneous distribution of cells. Preparations may feature high background or a low signal to noise ratio due to problems with the staining procedure or contain artifacts such as large algae that impair successful imaging of bacteria. Another source for low quality images can be the spatial unevenness of some FOVs, especially when high magnification objectives are used. This may affect both, an entire preparation or only small areas thereof. Finally, failures in the automated image acquisition procedure, such as inappropriate exposure times or autofocus errors, may lead to low quality images even from flawless preparations.

It seems important that such low quality images should not be further analyzed, because they decrease accuracy and reproducibility of cell counting routines and may lead to unpredictable results. It is therefore necessary to inspect autonomously acquired images and to remove low quality images from the data set. Autonomous image acquisition may provide several thousand images within a few hours. The manual quality control of image data is a time consuming and tedious activity so automation of this process would be highly advantageous.

Although in most cases it is easy for a human observer to decide whether an image is of high (HQ), medium (MQ) or low quality (LQ), automation is not trivial. It is generally
difficult to find reliable, measurable parameters and simple mathematical rules to assess image quality. On top of that, an entire image has to be classified as LQ even if only a part of it is of low quality, e.g., out of focus. In fact there are several categories of low quality images (Fig. 1) and even human observers may face difficulties to clearly define them.

Machine learning methods provide a strategy to recognize such complex patterns and make decisions based on learning from classified data. The availability of large amounts of images offers the possibility to rapidly generate human made training sets and favors the application of such methods. One such approach is the artificial neural network (ANN), a type of mathematical models inspired by the structure and behavior of animal neurons (8-10). They consist of interconnected layers of artificial neurons and have been successfully used to address a variety of scientific problems, from face recognition (11), protein phosphorylation site prediction (12), tumor diagnosis (13) to the classification of bacterial morphotypes (14). ANN analysis has also found application in industrial quality control, such as in machine vision based grading of apples (15) or cherries (16).

We developed a computer program that performs fully or semi-automated quality control of autonomously acquired microscopic images of fluorescently stained bacteria. The program includes an image viewer functionality and allows manual and automated sorting of images. Automation was achieved by low level image analysis and the implementation of a custom trained ANN. The algorithms for image processing are described in detail, and the program and the documented source code (VB.NET) are provided under General Public License.
Materials and Methods

Biological samples, microscopic system and images

Preparations subjected to autonomous imaging were produced in studies of bacterioplankton ecology in freshwater or marine ecosystems (5-7). Briefly, aquatic bacteria were filtered onto polycarbonate membrane filters and fluorescently stained. All cells were stained with a nucleic acid dye (4′,6-diamidino-2-phenylindole (DAPI) (17)). Specific phylogenetic staining was performed by fluorescence in-situ hybridization (FISH) (18,19). On some of the preparations, microautoradiography (MAR) was additionally performed for assessment of bacterial activity (20). Automated image quality assessment was exclusively performed on the images of DAPI stained cells (primary image).

Autonomous image acquisition was performed on a high-throughput imaging platform based on a motorized microscope (AxioImager Z.1, Zeiss, Germany). Functionality for autonomous imaging was implemented in Visual Basic for Applications module within AxioVision (Zeiss, Germany) (M. Zeder, unpubl.). Imaging was carried out using a CCD camera (AxioCam MRm, 12 bit grayscale, 1388 × 1040 px, Zeiss, Germany), a 63× Plan-Apochromat (NA = 1.4) objective, and a LED epifluorescence illumination device (Colibri, Zeiss, Germany) with four LEDs (365 nm, 450-700 nm, 470 nm, and 590 nm) in combination with a multi filter set (filter set 62 HE, Zeiss, Germany). Silver grains in the MAR preparations were imaged in bright field illumination.

Images were stored in the JPG format (grayscale, 8 bit). A fixed number of FOVs were imaged for every preparation, and the file naming was defined as follows: variant A: XX_YY_Z.JPG, or variant B: XXXYYYZ.JPG, where XX or XXX denotes the number of the preparation, YY or YYY the number of FOVs on that particular preparation, and Z represents the fluorescence channel (D: DAPI, P: FISH-Probe, T: MAR). The image quality assessment program is able to handle both naming variants.

Image test set

For training and testing of the ANN for automated image quality assessment, 25000
DAPI images were randomly chosen from the roughly 250000 image sets acquired in 2008 by different scientists at our department. The 25000 images were manually assigned to three different quality categories by one of the co-authors. The HQ category contained perfect images whereas the LQ category contained images that should not be subjected to further image analysis. The remaining images were assigned to the MQ category. Training subsets consisting of 33, 366 and 2200 randomly chosen images of each category were created. The images from each category were mixed in a regular systematic manner in the training set: a HQ image was always followed by a MQ image which was always followed by a LQ image. A validation subset with the same number of images was created accordingly.

Image processing, feature extraction and ANN design

Image processing for quality assessment was realized in VB.NET by the direct memory access method to efficiently retrieve pixel intensities of an image and transferring them into a two-dimensional array for further calculations. In order to address quality issues related to FOV unevenness or inhomogenous image quality, the image was subdivided into 9 or 16 rectangular parts referred to as subimages (SI). For evaluation purposes, the routine was also tested without image subdivision. Each image or SI was subsequently processed to extract three features for input to the ANN (Fig. 2). These features were the mean gray value (MGV), a cell density measure (CDM) and a background inhomogeneity measure (BGI).

The MGV was calculated as the sum of all pixel intensities $i(x,y)$ in a SI divided by the number of pixels in that particular SI:

$$MGV = \frac{1}{x_{\text{max}} y_{\text{max}}} \sum_{x} \sum_{y} (i(x,y)),$$  

(1)

where $x_{\text{max}}$ and $y_{\text{max}}$ were the number of pixels in x and y direction, respectively.

CDM and BGI were calculated using every second pixel scan line in x direction in order to increase processing speed. These features are similar to the focus algorithm described by Brenner et al. (21) but differ in that they apply a threshold ($T = 20$) for assessing large (CDM) and small (BGI) pixel intensity gradients. CDM was calculated as
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\[ CDM = \sum_x \sum_y (\Delta_{CDM} - 2 \cdot T)^2, \]  

(2)

where \( \Delta_{CDM} \) is equal to \( |i(x, y) - i(x + 2, y)| \) if this term is > 2 \( T \), else \( \Delta_{CDM} \) is 2 \( T \).

BGI was calculated as:

\[ BGI = \sum_x \sum_y (\Delta_{BGI} - T)^2, \]  

(3)

where \( \Delta_{BGI} \) is equal to \( |i(x, y) - i(x + 2, y)| \) if this term is > \( T \) and < 2 \( T \), else \( \Delta_{BGI} \) is \( T \).

Prior to input to the ANN, the values of each feature from the subimages were normalized and sorted in ascending order to create a position invariant input (Fig. 2). Data rescaling to the interval \([0.1, 0.9]\) has been shown to be optimal (22). Normalized values \( V_{\text{Norm}} \) were calculated from the values \( V \) as follows:

\[ V_{\text{Norm}} = 0.8 \cdot \min(V, V_{\text{max}}) / V_{\text{max}} + 0.1, \]  

(4)

where \( V_{\text{max}} \) was 255 for MGV and \( 5 \times 10^6 \) for CDM and BGI, respectively.

The ANN was implemented in VB.NET as a feed-forward network with three layers, an input, a hidden and an output layer. A sigmoid activation function was used to normalize neuron output values. The number of neurons in the input and the hidden layer was varied to determine optimal performance. There were three neurons in the output layer, one for HQ, MQ and LQ, respectively. The back propagation of error method was used to train the net. The ANN was trained to yield output values of 0.9 for a positive result and 0.1 for a negative result.

\textbf{ANN training and testing}

Different combinations of numbers of hidden neurons, training cycles, degrees of image subdivision and numbers of images in the training set were used to create a multitude of ANNs. The numbers of training cycles were 50, 100, 500, 1000, 5000, 10000, and 20000. The number of hidden neurons was chosen according to the number of input values (i.e., input nodes) that were dependent on the degree of image subdivision. ANNs for the analysis of entire images (no subdivision, 3 input nodes) were trained with 5, 10, 15, and 20 hidden nodes. ANNs for image subdivision into 9 SI (27 input nodes) were trained with 30, 50, 70, and 90 hidden nodes and ANNs for image subdivision into 16 SI...
(48 input nodes) were trained with 60, 80, 100, and 140 hidden nodes. All ANN variants were trained in triplicates. The resulting ANNs were subsequently tested against (i) the corresponding training subset, (ii) a validation subset consisting of 2200 images per quality category that were not present in the training subset and (iii) the entire set consisting of 25000 images (data not shown). The ANN with the highest correct identification rate was finally implemented in the newly developed program (see below).

**Performance comparison of humans and the optimal ANN**

The performance of six scientists was compared to that of the ANN. 250 randomly chosen images were assigned to the three categories HQ, MQ, LQ both by humans and the ANN. The classification by the scientists was repeated after several weeks to assess its reproducibility. The assignments were compared pairwise among the six scientists and to the ANN. In addition, the time required for classification was also considered. The image set is available for download (http://www.technobiology.ch).

**Influence of low quality images on cell counting precision**

In order to assess the influence of the presence of LQ images within an image set on the cell counting precision, six preparations of DAPI stained bacteria were imaged with the instrumentation described above. From each preparation, 60 HQ and 60 LQ images were used to create image sets containing different portions of LQ images. For this, different numbers of LQ images were added to the core set of 60 HQ images to obtain sets with 0, 10, 20, 30, 40, and 50% LQ images. The image sets were subjected to an automated cell counting routine developed in AxioVision VBA (M. Zeder, unpubl.). Briefly, images were subjected to a median filter and segmented by the 'Dynamic Thresholding' routine as implemented in AxioVision. Binary objects were then detected and counted, whereas too small and too large objects were excluded. Mean cell counts on the image sets were statistically analyzed by one way ANOVA to assess the level of acceptable portion of LQ images in a set. In addition, the mean coefficients of variation (CV) in sets with different portions of LQ images were also compared statistically.
Programming strategy, ANN implementation, and program description

The program, subsequently termed “Automated Image Quality Control” (AIQC), was developed in Visual Basic .NET (Microsoft) using an object oriented programming approach. The program offers to browse the images which are automatically sorted by preparation and FOV number by keyboard commands. Image sets can be manually selected and sorted into a different directory. The implemented default ANN allows for automated detection of either LQ or of MQ and LQ images. In order to extend the ANN functionality to other kinds of images than the default ANN was trained for, it is possible to create, train and save novel ANNs based on user-specific classification of images. The program can be run exclusively by command line arguments to facilitate its integration into an automated workflow. The program and the documented source code are available under General Public License in the supplementary material and at http://www.technobiology.ch.

Results

ANN optimization

Altogether, 420 ANNs with different combinations of parameters were created to find the optimal configuration. The categories HQ and MQ were merged into a single category 'good' to obtain dichotomous data. The percentage of correct classification was determined for both the training and the validation set (Fig. 3) by division of the number of correctly identified images by the sum of all images.

Training iterations and hidden neuron numbers. All ANN variations (Fig. 3 A-E) were trained with different numbers of training iterations, ranging from 50 to 20000. The correct identification rate on the training set increased steadily with the number of training iterations (Fig. 3 A-E, left panel side). However, on the validation set, there was an optimum reached (Fig. 3 A-E, right panel side) at 500 to 1000 iterations. All ANN variations featured four different hidden neuron variants. In general, higher numbers of hidden neurons lead to slightly higher correct identification rates, but at the expense of longer computation times.

Degree of image subdivision. The ANNs based on images without subdivision yielded a maximal correct identification rate of 82.1% (Fig. 3 A) on the validation set. Subdivision
into 16 SI lead to a maximum of 91.1% (Fig. 3 B). The overall maximum was achieved by subdivision into 9 SI and accounted for 93.7% of correct identification (Fig. 3 E).

**Training set size.** In order to assess the influence of the training set size on the performance of the resulting ANNs, three different set sizes were tested (Fig. 3 C-E). By using 33 images per category (HQ, MQ, LQ) a correct identification rate of 80.4% was achieved. 336 images per category yielded 92.2% of correct identification. The overall maximum of 93.7% was achieved with 2200 images per quality category.

**Optimal configuration.** Considering the correct identification rate and the rate of false positive and false negative identifications of all tested variants, the ANN based on nine SI, 90 hidden neurons and 1000 training iterations on a set of 2200 images per category was found to be optimal. The best of the three replicate ANNs was implemented in the AIQC program. The overall correct identification rate of this ANN on the entire set of manually classified images (n = 25000) was 94.0%.

**Performance comparison of humans and the ANN**

**Correlation between different humans.** Pairwise comparisons of image classification by humans (n = 6) revealed a mean correlation of 90.5 ± 4.2% (min = 81.6%; max = 97.6%).

**Reproducibility of humans.** When humans classified the same set twice, they agreed with themselves on 92.3 ± 2.5% (min = 88.4%; max = 95.6%) of the images.

**Correlation between humans and the ANN.** Pairwise comparison of classification between humans and the ANN showed a mean correlation of 88.5 ± 2.7% (min = 82.8%; max = 90.8%). Statistical analysis (one way ANOVA; Post-hoc test by Tukey’s method) revealed that none of the three above mentioned means was significantly different.

**Agreement of the ANN on the human consensus set.** A consensus set of images where all six humans agreed was compared to the ANN’s decision. 78% of all images were identically classified by all humans. 96.4% of the images from this consensus set were equally classified by the ANN.

**Classification speed.** Humans classified 0.85 ± 0.11 images per second whereas the AIQC program processed about 10 images per second on a state of the art personal computer.
Influence of quality control on counting precision

The presence of LQ images in a set lead to significant increase of the coefficient of variation (CV) of cell counts from a single preparation (Fig. 4) already when 10% of the images in the respective set were LQ. The mean cell counts on preparations were more robust against the presence of LQ images. Significant differences were observed only at proportions of 30% (n = 4) to 40% (n = 2) LQ images (one way ANOVA; Post-hoc test by Tukey’s method). The application of the ANN to the image data set with 50% LQ images correctly removed the majority of LQ images, so that the resulting mean cell counts as well as CVs were not significantly different from the original set of HQ images only.

Discussion

Image quality control is an important issue in automated imaging platforms (1). Low quality images need to be removed from data-sets prior to image analysis, because they decrease the counting precision and accuracy, as experimentally shown for bacterial cell counts (Fig. 4). It should be noted that these results are conservative error estimates, as the effects of LQ images on data derived from double or triple staining might be considerably more drastic. Automated removal of low quality images is thus of high advantage. On one hand, it significantly accelerates the quality control process. On the other hand, decisions are more objective and reproducible, as illustrated by the comparatively low reproducibility of human assessment.

ANN input data. The input data for an ANN are of crucial importance. The information retrieved from the image should not be too specific and should be invariant to rotation and position. Moreover, it should allow recognizing a LQ image if only a small part of the image is of low quality (Fig. 1 F). The strategy of dividing an image into separately analyzed SI can be a powerful approach for identifying FOV unevenness during autofocusing (23). Image subdivision into 9 parts also significantly improved the correct identification rate in image quality control, compared to the analysis of entire images (Fig. 3). However, subdivision into 16 parts did not result in further improvement. A possible explanation could be that higher degrees of subdivision increase the number of input
values, thereby rendering the learning process more complex, and the input values *per se* are less meaningful, as they represent smaller image areas. Alternatively there may be an optimal relationship between the size of the SI and the image features of interest (in our case, fluorescently stained bacteria).

In order to assess the quality of a SI, three features were extracted: the mean gray level intensity (MGV), a measure for cell density (CDM) and a measure for background inhomogeneity (BGI). On the whole image level, the parameter MGV is sensitive to some types of unfocussed images or too long exposure times (Fig. 1, H and E). The presence of single large bright objects (Fig. 1 G) can be detected by comparing the MGVs on the 9 SI. The main problem of this parameter is that it is not able to detect empty fields, because cells, even though they feature high gray level intensities (approx. 100 to 255) compared to the background (approx. 20 to 40), only cover a small part of the total image area. Thus, most of the image pixels represent background (> 98%) and the presence or absence of cells cannot be deduced by the MGV parameter. Therefore, the presence of cells was assessed by the CDM feature which is a custom modification of the Brenner Gradient algorithm (21). It quantifies gray level gradients by measuring the squared difference of the intensity of a pixel and its neighbor two pixel ahead on a scan line. Fluorescent objects typically feature large gradients along their contour. In order to determine thresholds, we analyzed the gradients of both background and cells on HQ and MQ images (n = 19638, Fig. 5 A). Only 0.1% of all gradients in the background of images were larger than 40 and 0.3% of the gradients were larger than 20, respectively. As an indicator for cells we set a threshold of 40 to exclusively detect strong gradients. On the whole image level, this allows a reliable distinction between empty images and images containing cells. Moreover, the inhomogeneity of the distribution of cells within the image can be assessed by comparing the CDM values of the individual SI.

For the counting and measurement of the size of cells, the mere presence of fluorescence signals from objects is not sufficient, as they must be well focused, too. While it is easy to specify the in-focus image of cells in a z-stack of images (e.g., during autofocusing), it is much more demanding to derive focus information exclusively from a single image (1). This is even more difficult if it has to be specified *a priori* before actual object detection, i.e., without knowledge of the geometrical and densitometrical properties of the objects. Small objects in the sub-micrometer range, such as many aquatic bacteria, exhibit a halo when they are imaged out of focus, because the image of a point source of
light is a three-dimensional diffraction pattern (24). The intensity of the halo is lower than
the fluorescence intensity of the object itself. These weak gray level intensity gradients in
close proximity to fluorescent objects are thus characteristic for unfocussed images. We,
therefore, modified the CDM parameter by applying another threshold (the range 20 to 40)
to exclusively measure weak gradients, rendering this new parameter (BGI) sensitive to
the halo pattern. A linear combination of two parameters, e.g., their difference, gives a
rough measure for the 'focussedness' of objects on an image (Fig. 5 B), allowing for
detection of unfocussed or partially unfocussed images (Fig. 1 F, Fig. 2) by ANN analysis.
A similar approach, but relying on prior cell detection and subsequent analysis of the pixel
intensities around the cell boundaries has recently been described for successful detection
of unfocussed cells (1).

**ANN Training.** Comparison of different sizes of training sets showed that larger training
sets enhance the performance of the ANN (Fig. 3 B, J, L), but also that the gain is greatest
when increasing the training set size from 33 to 366 images per quality category.
Especially that equal numbers of images per category and regular mixing of those
appeared to be an important issue for successful training (data not shown).

Several variants of feed forward back propagation ANN were trained. The two main
variables were the number of training iterations and the number of hidden neurons, and
the former was found to be of greater importance. The effect of overtraining was observed
after more than 1000 iterations: the rate of correct identification still increased on the
training set whereas the correct identification rate on the validation set cumulated on 500 –
1000 iterations and subsequently decreased (Fig. 3 E).

**Overall ANN and human performance.** The ANN optimization resulted in a maximal
performance of 94% of correctly classified images. However, the correct identification rate
is based on a training set that had been manually classified by a human expert.
Comparison of the classification performance of different humans showed that the same
set of images (n = 250) was differently classified by different humans, with a mean
pairwise agreement of about 91%. If humans classified the same set twice, the
reproducibility was slightly but not significantly higher (92%), reflecting the fact that there is
a fraction of images that cannot be unambiguously assigned to either category even by the
same individual. The pairwise agreement of the ANN to the humans was not statistically
significantly different from the pairwise agreement between humans. We, therefore,
conclude that the ANN can be trained with high precision to a specific training set. This is
supported by the finding that 96% of the images from the core set (i.e., all the images that were identically classified by six humans) were also classified likewise by the ANN. Apparently, humans are not able to correctly assign a certain proportion of images to a quality class, and for these images, the human classification is highly subjective and not reproducible. As the training and testing sets for the ANN was created by a human, this uncertainty is also inherently present in the ANN itself. We cannot expect the ANN to behave more precise than the human ability for correct assignment.

Altogether, the ANN approach for an automated quality assessment of microscopic images of stained bacteria proved to be successful, both with respect to correct identification rate and to classification speed. The program offers the possibility to implement differently trained ANNs in order to classify other types of images than described here. The authors are therefore confident that the program and the underlying strategies can be applied for quality control in other image based screening platforms.

**Future perspectives:** During this study it has become clear that any decision about image quality will strongly depend on the purpose of the subsequent analysis and on the type of biological sample. Although the here described ANN performs well on the tested image set, it may fail on other types of samples, e.g., from habitats that feature higher loads of organic material (resulting in higher image background inhomogeneity), or for larger planktonic organisms (algae). Our solution for this inherent shortcoming was to provide the possibility to train user or sample specific ANNs within the program. Still, the three parameters we choose eventually may not be adequate any more for entirely different image types and thus might need to be replaced or supplemented with more specific ones.

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**Legends to Figures**

Figure 1:

Figure 2:
Schematic representation of the image processing workflow: Subdivision of the image into nine parts and determination of three features (mean gray value: MGV, background inhomogeneity: BGI, cell density measure: CDM) per sub image. Artificial neural network (ANN) input is generated by normalization and sorting of the measured features. The ANN consists of 27 input (Nr. 1-9 for MGV, nr. 10-18 for BGI, and nr. 19-27 for CDM values), 90 hidden and 3 output neurons. The ANN output for the depicted image is maximal on the low quality (LQ) output neuron (0.910) and the image is then correctly identified as LQ image.

Figure 3:
Performance of different ANN configurations. For differently configured ANNs, the correct identification rate (A-E) on both, training (T) and validation (V) set is plotted against increasing training iterations. In F, false positive and false negative identification rate on the validation set is plotted against the number of training iterations. Each data point represents a triplicate of the same ANN configuration (Error bars show standard deviation of the triplicates). The hidden neurons number (symbols), the degree of image subdivision (SI: 0, 9, 16) and the training set size (33, 336, 2200 images per quality category) was varied.
Figure 4:

Precision of cell counting in relation to the fraction of LQ images in an image set. A: Mean cell counts per image of image sets with different portions of LQ images of one preparation (60 HQ images plus 0 – 60 LQ images). B: Coefficients of Variation of cell counts of 6 image sets with different portions of LQ images. Asterisks above boxes indicate significantly different means (ANOVA, Post-hoc test by Tukey’s method) from the image sets containing no LQ images (white box).

Figure 5:

Changes of the image quality measures (CDM and BGI parameter) along a z-stack of 20 images. Sections of the images 6 to 15 are depicted on the right. Normalized measures of CDM and BGI on every image are shown as full and dashed lines, respectively. The gray area on the plot resembles the difference of BGI and CDM.
Zeder et al, Figure 2
Zeder et al, Figure 3
Zeder et al, Figure 5