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Video microscopy as an alternative method for somatic cell count in milk

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This paper presents an alternative method to estimate somatic cell count (SCC) in cows' milk. SCC is an important indicator in the detection of inflammatory reactions within the udder in cows and Direct Optical Microscopy (DOM) is the present reference method for SCC but, owing to its dependence on human operators, it is extremely costly, time-consuming and potentially subjective. The industrial method of choice is Epifluorescence (EF), which has the potential for impressive throughput and acceptable precision, but requires huge inversions and handling of highly toxic reactives and waste. In this paper, an advantageous method that involves application of a low-cost Video Microscopy (VM) system is analysed and discussed, including a comparison between DOM and VM, and an example of application of both methods to evaluate EF counts. We conclude that VM is sufficiently precise and very cheap to implement and operate.

Keywords: Direct optical microscopy, epifluorescence, cows' milk.

Evaluation of somatic cell count (**SCC**) in fresh cows' milk has implications for milk quality, productivity, animal health, and trade issues. These cells increase levels of lipolysis and proteolysis, which affect quality and shelf-life of fluid milk (Ma et al. 2000), reduce yield and quality of cheese as a result of decreased curd firmness and fat and casein retention (Politis & Ng-Kwai-Hang, 1988) and worsen sensory quality. SCC is an important indicator of the degree of subclinical intramammary infection in cows, which is considered to be the most economically important degree of mastitis because of its long-term impact on milk yield (Deluyker et al. 1993; Harmon, 1994; Haenlein & Hinckley, 1996).

Direct Optical Microscopy (DOM) has been adopted by the International Dairy Federation (IDF) as a reference method for SCC through IDF Standard 148A (IDF, 1995). This standard describes the procedure for sample preparation, dyes, microscope spatial calibration and cell count. This method is highly accurate, but its throughput is poor and it requires highly qualified personnel to avoid subjectivity, which may reduce count repeatability, imposing short counting sessions for a single operator. Wherever a high precision system for evaluation of SCC or a reference procedure is required, such as in laboratories performing calibration or validation of SCC and/or mastitis detection equipment, implementation of the DOM reference method may place heavy demands on staff. Furthermore, criteria followed for cell identification cannot be stored; thus, changes in criteria require microscopy slides to be analysed again.

Some of the drawbacks described above may be addressed by combining a microscope, a CCD camera allowing eyepiece-camera parfocality and parcentricity, and a monitor/receiver in a Video Microscopy (VM) system. Microscope output is handled by a computer-operated image acquisition and processing system. This technique is extremely well suited to high-throughput automation and has reduced staff requirements for sample preparation. Video cameras attached to microscopes have been largely used for particle identification and classification because of the ease of processing, analysing and storing images. It requires no specialised hardware and there is plenty of software, open-source or otherwise, ready for the job and the tool is available for any small-scale laboratory. This paper describes an automated VM system for SCC. The objectives of this study were to assess the performance of, and present results for a VM system for cows' milk compared with the reference DOM method. To check the capability and accuracy of this method both DOM and

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VM were used to evaluate a set of epifluorescence (EF) counts.

Materials and Methods

Milk sample collection

Milk samples were collected from 80 Holstein-Friesian cows at a regional research council dairy farm (CIATA, Asturias, Spain) during 2000 and 2001. Milk samples (60 ml) were taken from individual cows at morning milkings and were kept refrigerated without preservative, to be processed within 4 h of collection, and analysed later in the evening of the same day.

Epifluorescence

To compare methods, EF counts for samples collected from the same cows and days were obtained from the herd records of the Official Milk Testing organization (ASCOL, Asturias, Spain). Milk testing teams collect azidiol-preserved, individual cow samples from the evening milking, and keep them refrigerated. Samples are analysed at the regional milk testing laboratories for SCC with an epifluorescence-based Fossomatic 5000 system (Foss Electric A·C, DK-5000, Hillerød, Denmark) within 24 h of collection. These laboratories undergo monthly IDF compliance and aptitude checks performed by the Spanish Department of Agriculture. Figure 1 describes the sample collection.

A total of 1248 samples were collected for the purpose of setting up the VM procedure, with 76 samples analysed both by VM and by DOM, 252 by VM and EF, and 129 by DOM and EF. No repeated EF counts were available for official test samples and thus repeatability of methods could not be calculated.

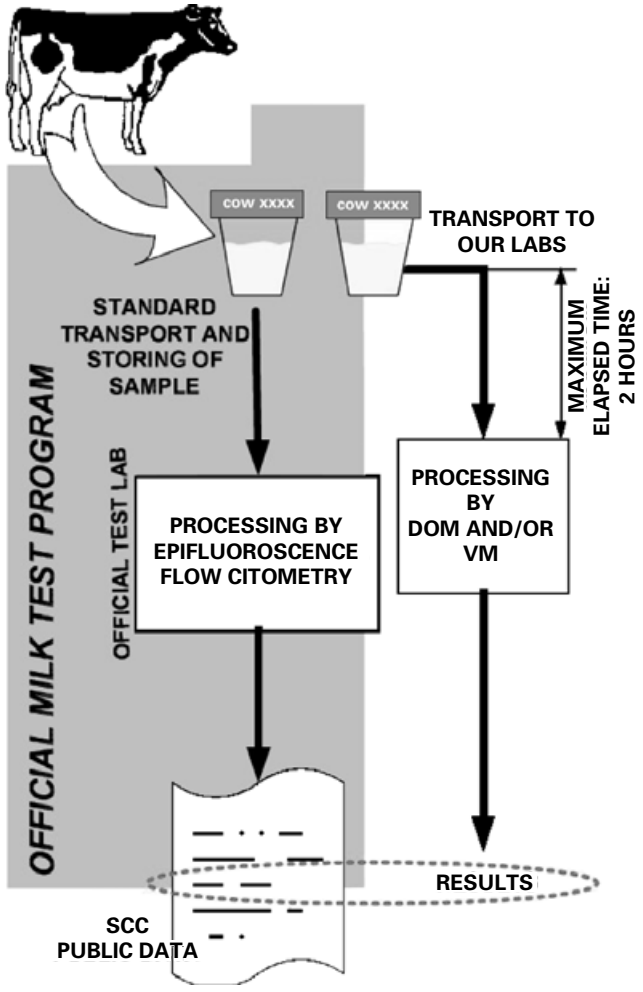

Fig. 1. Sample collection.

Cell counting

Staining. Milk samples were kept at 5 °C and processed within 4 h of collection. They were heated to 40 °C in a water-bath for 15 min before being cooled to 20 °C by stirring. A 1-cm^2 area of a degreased microscopic slide was then covered with 10 µl of sample and dried in an oven at 60 °C for 4 h in a dust-free environment, following the guidelines in IDF Standard 148A (IDF, 1995). Minor departures were taken from this Standard, such as coating microscopy slides with poly-L-lysine in order to prevent milk smears not adhering evenly to microscopic slides. Additionally, in an attempt to reduce edge effects, the sample covered an 1-cm × 1-cm square area as in haematology slides, instead of a 20 mm × 5 mm area as indicated in the IDF Standard.

Quality check. Extreme care must be taken in the preparation step as samples with defective adhesion, as well as samples with uneven cell distribution should be rejected. Sample quality is evaluated by visual inspection with the 4 × objective before image acquisition.

Video Microscopy

The VM system for cow milk SCC consisted of a personal computer (PC), a high resolution monitor, a video acquisition card (Video Acquisition Card IMAQ PCI-1407, National Instruments Corporation, Austin, TX 78759-3504, USA), a high resolution B/W CCD camera (1.3 Megapixel Camera JAICV-M1, JAI A·S, DK-2600 Glostrup, Denmark), and an optical microscope (trifocal optical microscope PB4161, EUROMEX B·V, 6836 BD Arnhem, The Netherlands). A wide choice of commercial CCD cameras, PC acquisition cards, and video processing software permits flexible implementation of the VM method using any matching camera-card-software combination. Figure 2 shows a VM working system.

The VM method procedure comprises three steps:

Spatial calibration of the system
Milk sample preparation: staining and quality check
Image analysis: capture and processing

The core of the VM method, depicted in Fig. 3, lies in the image acquisition step and its processing by software.

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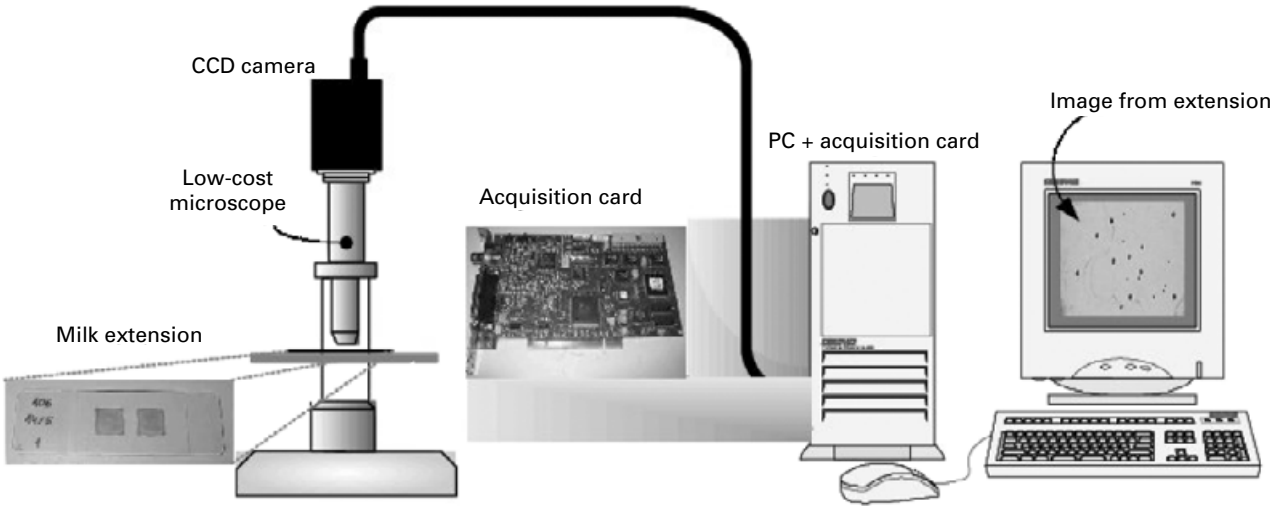

Fig. 2. Video Microscopy system comprising a PC, microscope and CCD camera.

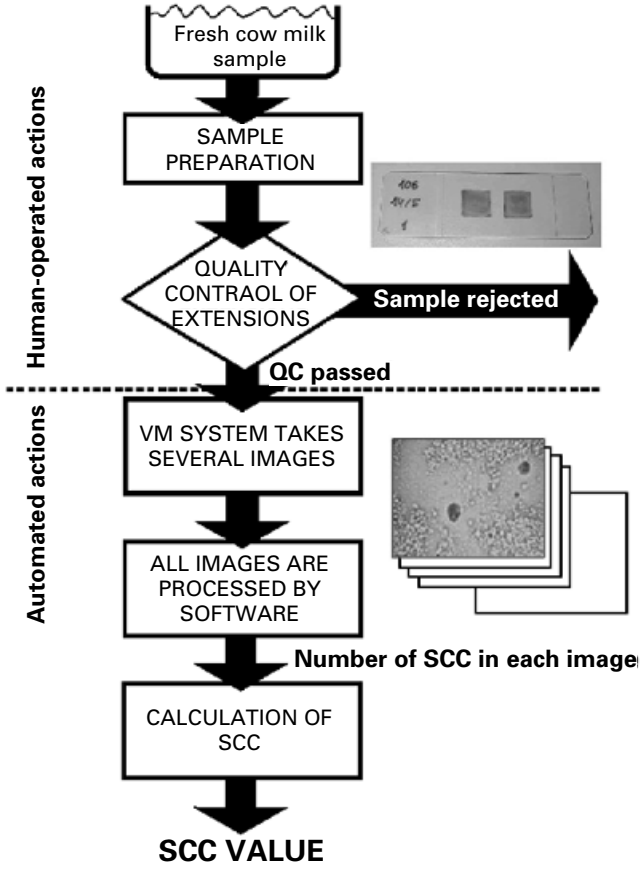

Fig. 3. The Video Microscopy method from cow to SCC.

Milk sample preparation steps are identical to those used for DOM.

Spatial calibration of the system. To convert numbers of observed cells into SCC values, measured in cells/µl, a microscopic factor (MF) is required, defined as the number of microscopic fields per microlitre. Under the particular conditions of our set-up, MF is 96·926 for the 40× objective plus camera lenses (Grillo et al. 2001). Adhesive phenomena and formation of clusters hinder the sorting of individual cells at very high counts, above 20 per field, and may require a 100× objective. This case was not investigated further as such high counts, corresponding to SCC>2×10^3 cells/µl, are beyond the range of useful measurement.

Capture. Microscopic fields were located and focused upon with the 4× objective. Once checked, the 10× objective was used for locating the upper left corner of the slide, allowing for a margin to avoid edge effects. It must be noted that focus should be centred on the nucleus shadow, as projected on the upper cytoplasmic surface by the light source, for better contrast. Image acquisition proceeded with the 40× objective and was sequential, row-wise, with no overlapping fields. Coordinates for the next field may be set either manually or with a stepper engine. The number of field images to be acquired per sample is addressed below.

Processing. Once captured, somatic cells are counted by identifying particles of certain morphology and grey level intensity, and then averaged across the slide after outlier editing. Microscopic field counts are converted into SCC in cells/µl by application of a MF as obtained from the spatial calibration step.

This procedure was implemented through an automated batch process dependent on certain parameters, and yielded a series of properties for each particle (surface, perimeter, shade of grey, etc.) in a spreadsheet table. Image analysis was performed with a closed-source program (IMAQ Vision Builder v1.0, National Instruments,

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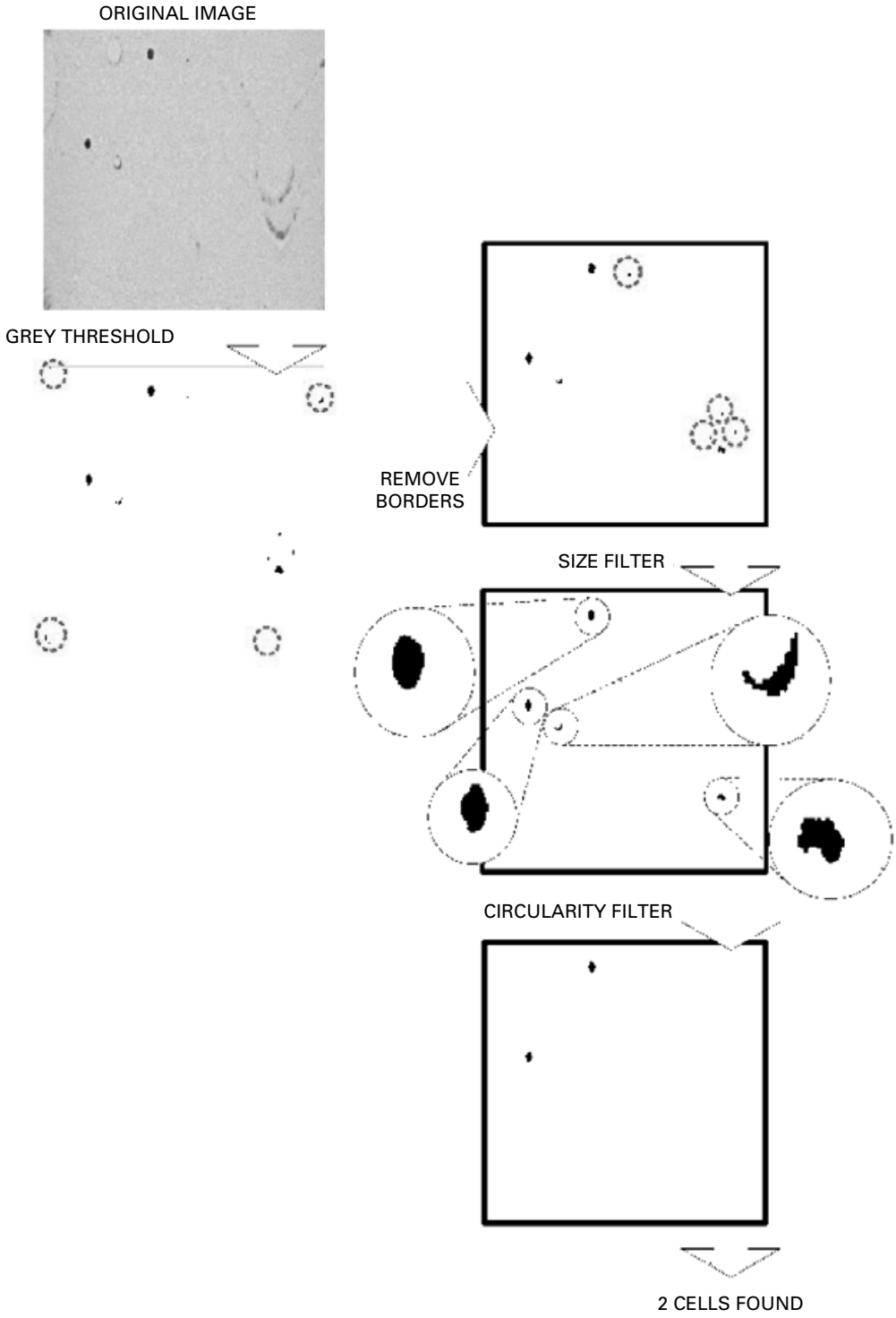

Fig. 4. Image processing steps for the automated identification of somatic cells in cow milk: 1) Turn background into white by setting an adequate grey threshold. 2) Particles lying over borders are removed to avoid repeated counts. 3) Particles within somatic cell size ranges are kept. 4) Shape criteria are applied.

2000). Several open-source code alternatives are available as well and a thorough review may be found in Karasik (2003). This information may lead to more sophisticated cell analysis. Figure 4 shows the steps involved in the automated identification procedure. Two parameters are required for processing the images:

Surface: ranging from 25 to 300 μm^2. Such broad range must cover surfaces for most immune cells present in milk,

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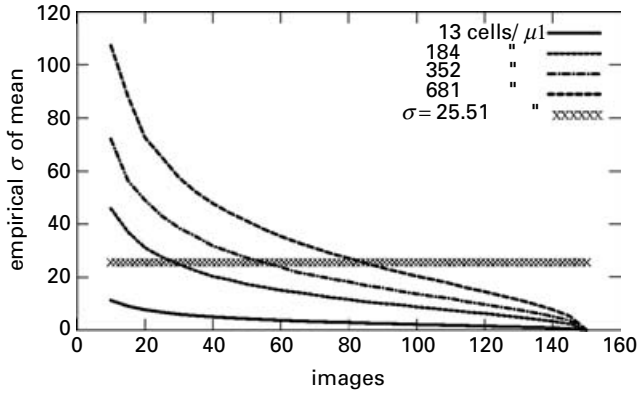

Fig. 5. Standard deviation for means based on different numbers of images in four milk samples with varying cell counts. The crossed-line indicates the suggested error limit of 50 cells/ml.

such as lymphocytes (25–100 μm^2), polymorphonuclear neutrophils (100–150 μm^2), and monocytes (130–300 μm^2) (Tizard, 2000).

Circularity factor: a Heywood factor is calculated as the ratio of particle perimeter to the perimeter of the circle of the same area. This factor takes a value of one for a perfect disk. Particles with a factor greater than 1·2 were rejected. This was to allow for distortions introduced by differences in image aspect ratio as captured by the CCD and as subsequently acquired by the computer video card. Under the set up described above, the CCD captured images of 5 : 4 aspect ratio while the video card acquired 1 : 1 images distorting both size and shape.

Statistical methods

Distribution of counts within samples was checked for normality with the Kolmogorov-Smirnov test. Bootstrap resampling was used to investigate the number of images to be analysed to attain a certain precision. The degree of agreement between methods was assessed with Bland-Altman plots of differences *v.* averages (Bland & Altman, 1986). Samples used for this study were actual measurements from a production herd and their distribution reflects the exponential nature of SCC. A log-scale for the x-axis provided more uniform plots, and relative differences ((A−B)/((A+B)/2)) were better suited for reflecting fit of ratio-scaled variables with very wide ranges. For these two reasons, an additional graph is shown in each case.

Agreement between methods was statistically tested by means comparisons and regression studies. A non-paired means comparison shows whether values produced by both methods lie within the same sample space, while paired means comparisons may detect the existence of a systematic bias of one method with respect to the other. Both paired and non-paired means comparisons were carried out by the non-parametric Mann-Whitney and Wilcoxon test (Wilcoxon, 1945), which is not affected by deviations from normality and is almost as powerful as the *t* test when normality holds. Logarithmic transformations were applied for the regression studies in order to improve normality of residuals, as the best Box-Cox normalizing transformation (Box & Cox, 1964) is not significantly different from a logarithmic transformation.

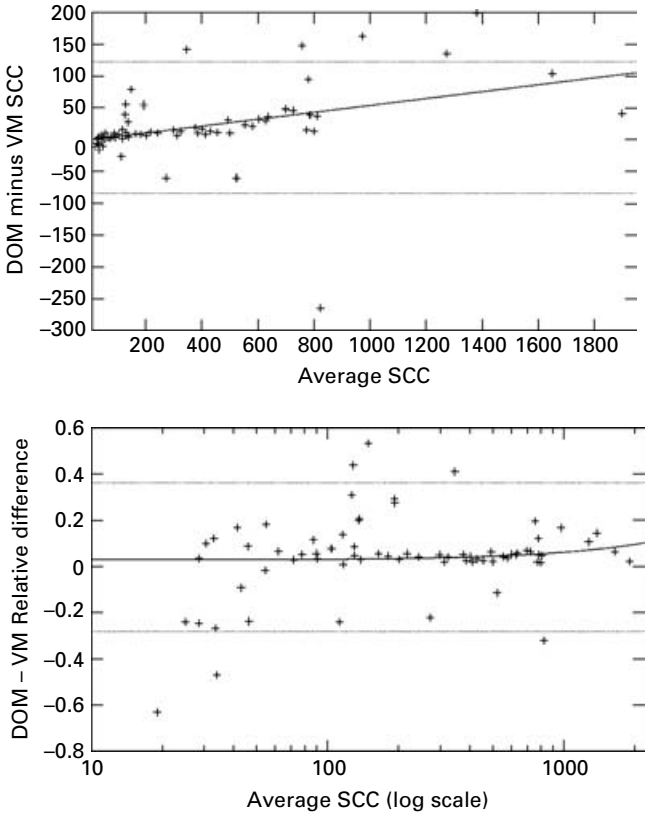

Fig. 6. Direct optical microscopy (DOM) *v.* Video (VM) counts. Figure (a) shows differences (DOM−VM) plotted against averages ((DOM+VM)/2), with 95% limits of agreement (broken line) and regression line. Figure (b) shows relative differences ((DOM − VM)/(DOM+VM)/2) plotted against log-scaled averages.

Two linear regression models were fitted in order to predict DOM results from EF and VM measurements, $DOM \sim \beta_0+\beta_1 EF$ and $DOM \sim \beta_0+\beta_1 VM$ respectively, thus comparing the adequacy of these methods by means of the study of regression parameters. In each case, we tested for a null intercept to check proportionality between methods with the hypotheses H0: $\beta_0=0$, H1: $\beta_0 \neq 0$, and for coincidence of measurements with the hypotheses H0: $\beta_1=1$, H1: $\beta_1 \neq 1$, when $\beta_0=0$.

Results

The number of images to be analysed per sample was investigated by means of bootstrap resampling of 10 000 iterations for each sample and for each number of images. Figure 5 displays the empirical SD for measures based on different numbers of images calculated as average

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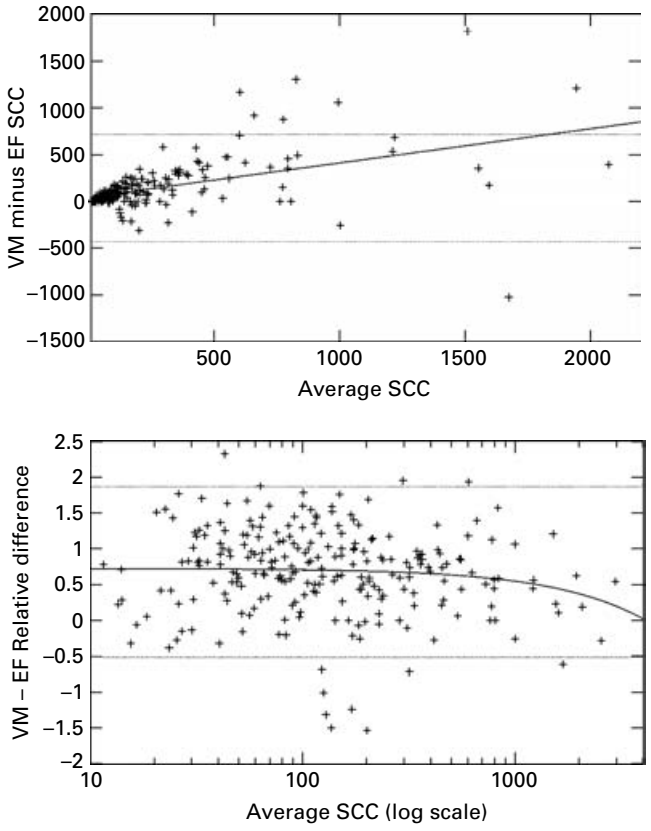

Fig. 7. Video (VM) *v.* Epifluorescence (EF) counts. The upper figure shows differences (VM−EF) plotted against averages ((VM+EF)/2), with 95% limits of agreement (broken line) and regression line. The lower figure shows relative differences, 2∗(VM−EF)/(VM+EF), plotted against log-scaled averages.

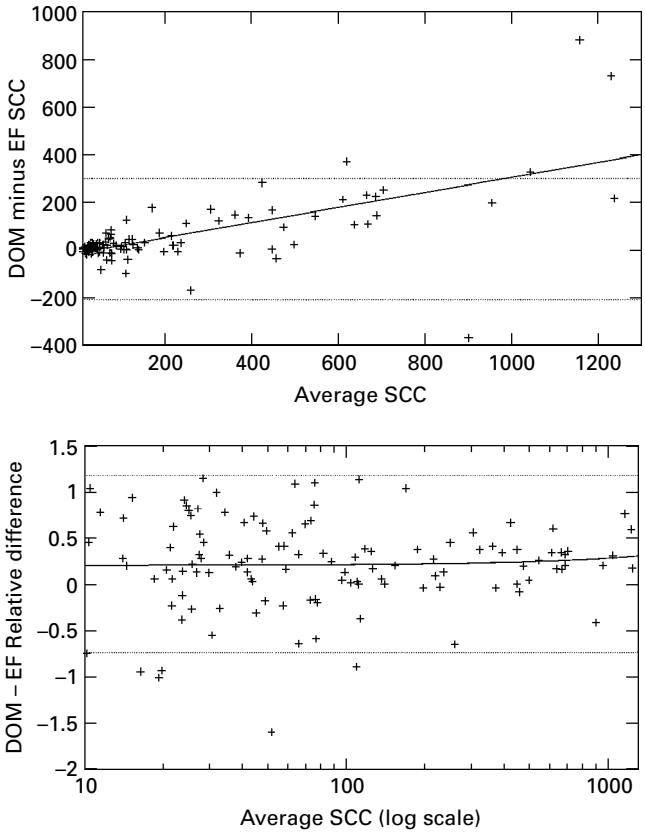

Fig. 8. Direct optical microscopy (DOM) *v.* Epifluorescence (EF) counts. The upper figure shows differences (DOM−EF) plotted against averages ((DOM+EF)/2), with 95% limits of agreement (broken line) and regression line. The lower figure shows relative differences, 2∗(DOM−EF)/(DOM+EF), plotted against log-scaled averages.

microscopic field counts multiplied by a MF of 96·926. Field counts passed the Kolmogorov-Smirnov test for normal distribution with a *P*-value of 0·063. It can be seen that, assuming normality and with a confidence level of 0·95, an error of ±50 cell/μl requires at least 80 samples. Higher counts showed greater errors and would require a larger number of images.

The most informative counts are those in the range from 100 to 1000 cell/μl, and correspond to counts valuable for diagnosis of intramammary infection. Classification of the health of a quarter was earlier based on a threshold of 500 cell/μl, but Hillerton (1999) has suggested that the threshold of 200 cell/μl provides better discrimination between infected and uninfected quarters.

Figure 6 shows Bland-Altman plots for 76 milk samples analysed with DOM and VM. SCC were obtained from 80 images per sample with VM, and from 300 images per sample with DOM. It can be seen that fit is best for counts in the most informative range. For counts >1000 cell/μl, DOM counts seem to be higher than VM counts, although not enough of such high counts were observed to allow a firm conclusion. The relative bias in measurements between the two methods is approximately 5% for the most informative counts, and does not exceed 10% anywhere in the range of counts. This bias should not be affected by the number of images analysed per sample.

Table 1. Results for comparisons of means and regression analyses between pairs of methods. DOM/EF stands for the model $DOM \sim \beta_0+\beta_1 EF$, and DOM/VM stands for $DOM \sim \beta_0+\beta_1 VM$. Estimated values and *P* values are shown for intercept (β_0) and regression slope (β_1). *P* values are enclosed in parentheses

TEST		DOM/EF	DOM/VM
Wilcoxon	non-paired	(0·1327)	(0·6950)
Wilcoxon	paired	(0·0000)	(0·0000)
H0: $\beta_0=0$	H1: $\beta_0\neq 0$	0·538 (0·0006)	−0·077 (0·3635)
H0: $\beta_1=1$	H1: $\beta_1\neq 1$	0·925 (0·0358)	0·960 (0·0065)
	R^2	0·8482	0·9808

To compare the two methods further, both DOM and VM were used on samples for which corresponding EF counts were available. Figure 7 shows a Bland-Altman plot for VM and EF counts, and it can be seen that the relative bias is near 0·5. The 95% limits of agreement

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are [−0·51,1·87] for relative bias, which distributes quite evenly along the log SCC scale of informative counts.

These data may be compared with the Bland-Altman plot for DOM and EF counts in Fig. 8. The 95% limits of agreement are [−0·74,1·18] for relative bias, averaging 0·223. Both DOM and VM present a slightly downwards skewed distribution for their difference from EF counts, meaning that it is more likely that microscopic counts would be lower than EF counts.

Agreement between methods was statistically tested by study of means and regressions. Table 1 shows results for means comparisons and regression analysis between pairs of methods.

Non-paired means comparisons failed to detect significant differences between the reference method, DOM, and each of EF and VM. As for the paired means comparisons, both comparisons resulted in significant differences, showing a systematic bias between methods as mentioned above.

The degree of agreement between methods was further investigated with a regression study. The determination coefficient R^2 for VM as a linear predictor of DOM is 0·9808, clearly higher than the R^2 for EF as a linear predictor of DOM which is 0·8482. Estimated values are shown for the intercept (β_0) and regression slope (β_0). The relevant hypotheses in this context were checked by testing H0: $\beta_0=0$, H1: $\beta_0\neq 0$, i.e., testing for a null intercept to check proportionality between methods and testing H0: $\beta_1=1$, H1:$\beta_1\neq 1$, for coincidence of measurements when $\beta_0=0$.

It was found that EF counts are significantly not proportional to DOM counts. VM counts seem to be proportional, but failed the second test ($\beta_1=1$), which indicates that a scale correction must be applied in order to get optimal predictions.

Discussion

Cell counting requires particles to be distinctly identified as cells. Lack of resolution to distinguish between complete and fragmented cells, at which EF excels, may constitute a source of error. This is probably shared by DOM and VM and may be addressed with enhancements in the image-processing step with no need to resort to nucleus-specific, albeit highly toxic dyes used in EF. Methylene blue staining makes out clearly the morphology of living cells but is not specific for nucleated somatic cells. Cytoplasmic particles are sligthly stained as well. This may not pose a problem as a source of bias for this study because Official Milk Testing mid-milking samples display few cytoplasmic particles. For a study of direct microscopy and staining specificity for cells and cytoplasmic particles in milk samples, see Gonzalo et al. (2002).

There are other sources of error specific to VM as it may be unable to identify shapes out of focus, i.e., those in the lower planes of the sample, and to identify overlapping cells. This may explain the downward bias with respect to DOM detected in samples with very high counts in which cell overlapping is more likely. A possible explanation lies in automated image processing being worse than human operators at singling out cells in clusters.

Fit is best for counts between 100 and 1000 cell/μl and this range corresponds to the most informative counts. Bias is almost constant for relative differences in this range of counts and in the order of 5%.

Determination coefficients from regression analyses show that predictions of DOM with VM are potentially better than predictions of DOM with EF. Use of raw VM counts leads to rejection of the hypothesis of $\beta_1=1$. VM counts could be easily manipulated to resemble DOM counts better by application of a linear transformation.

The method proposed for SCC in milk is sufficiently precise and does not suffer from the subjectivity of methods that rely on human operators for cell identification. This is so despite the fact that only 80 images were used for VM estimation of SCC, while 300 images were used for DOM estimation. Precision is maximal for the range of counts that include discrimination thresholds for bovine intramammary infection. For counts up to 1000 cell/μl, VM performs almost as well as DOM when compared with a third counting method such as EF. Images and the outcome of their analyses may be stored for later use, as well as additional cell properties that may lead to more sophisticated cell counts. This is achieved at very low implementation and operational costs, and the authors offer support to any team wishing to test this technique. The results presented above fully support the hypothesis that VM constitutes an advantageous alternative over DOM for precise estimation of SCC.

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