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Direct labeling of milk cells without centrifugation for counting total and differential somatic cells using flow cytometry

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ABSTRACT

Somatic cell count (SCC) in milk is an essential indicator for defining and managing udder health. However, analyzing differential SCC (dSCC) can be helpful in determining the type or evolution stage of mastitis. A high abundance of polymorphonuclear cells (PMN) is associated with acute mastitis; however, the status of a chronic disease is less well characterized. A method capable of analyzing SCC and dSCC can prove to be a helpful tool for monitoring the status of evolution of mastitis disease in a better way. Therefore, a new direct-flow cytometry method was developed to count and differentiate somatic cells in milk without the steps of centrifugation or washing, avoiding variabilities that occur due to enrichment or loss of specific cell types. In this new method, SCC is analyzed using the method of DNA staining with Hoechst stain, whereas dSCC are analyzed using specific antibodies targeting 2 main cell types associated with mastitis: PMN cells and antigen-presenting cells, which are associated with innate and adaptive immunity. Equivalent SCC values were obtained between the new method and the routine ISO 13366-2 method in a comparison of 240 raw milk samples. Furthermore, dSCC results were confirmed by microscopy after May-Gründwald-Giemsa staining in 165 quarter milk samples from healthy and diseased cows. The method was verified with fluorescence microscopy on the 2 targeted cell types and in raw milk samples. The newly developed method is independent of any instrument and can be further designed to differentiate other cell types and animal species by selecting appropriate antibodies.

Key words: total somatic cells in milk, mastitis, differential somatic cells in milk, flow cytometry

INTRODUCTION

Total SCC in milk is used worldwide to indicate udder health and milk quality and is routinely analyzed with ISO standard 13366-2 (ISO, 2006). Milk from diseased cows with SCC above the defined limits, which are 350,000 cells/mL for Switzerland, 400,000 cells/mL for the European Union, and 750,000 cells/mL for the United States (EU, 2013; Alhussien and Dang, 2018; EDI, 2020), is not suited for human consumption. With SCC above such limits, milk delivery is blocked, which causes significant economic losses to producers. Mastitis, which causes high SCC, represents a serious health problem of cows, even when SCC is often lower than the limit that is legally acceptable in cases of subclinical or chronic mastitis (Jadhav et al., 2018). Moreover, cell numbers and types in milk are dependent not only on the health status of the cows but also on their breed, lactation stage, and sampling (Leitner et al., 2000). The total number of cells in milk does not provide any information about the type of mastitis or evolution stage.

It is well known that acute mastitis is defined by a large proportion of PMN in milk. However, the distribution of macrophages and lymphocytes in healthy and subclinical mastitis samples is variable (Leitner et al., 2003; Paape et al., 2003; Schwarz et al., 2011; Damm et al., 2017). To better understand udder health, analytical methods for differential somatic cell count (**dSCC**) are required to understand the distribution of somatic cells and predict and, eventually, prevent mastitis. A mastitis screening is provided in a recently published method for dSCC, which is based on the method of DNA staining with acridine orange; however, information on the different cell types is not provided. The mastitis probability increases due to a more abundant presence of PMN in samples with increasing SCC (Damm et al., 2017). Several dSCC methods based on flow cytometry using specific antibodies have been developed. However, sample preparation in the methods published so far was performed on cells extracted from the original milk, using centrifugation and washing steps (Dosogne et al., 2003; Gunasekera et al., 2003; Koess and Hamann,

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2008; Schwarz et al., 2011) to reduce interferences of milk fat globules (**MFG**).

Due to differences in cell density, specific cell types tend to be enriched in the fat layer or lost during sample preparation (Paape et al., 2003), which results in alterations in the original distribution of particular cell types. Due to the complexity of the milk matrix, the goal of this study was to develop a new, direct-flow cytometry-based method, allowing the counting and differentiation of somatic cells in milk without the steps of centrifugation and washing to avoid such biases. Somatic cell counts were shown to be in good agreement by a method comparison between ISO standard 13366–2 (ISO, 2006) and the new method. In addition, cell differentiation was performed using specific antibodies for integrin α-M (**CD11b**) and major histocompatibility complex class II (**MHCII**). The CD11b is mainly expressed by most of the immune cells, such as monocytes, granulocytes (PMN), lymphocytes (B cells, T cells), and macrophages, present in milk but not by epithelial cells. The MHCII is mainly expressed by antigen-presenting cells (**APC**), such as monocytes and macrophages, and is at a lower level by B cells and T cells (Pilla et al., 2013; Lipski et al., 2017). As a confirmation of cell differentiation, single positive ($CD11^+/MHCII^-$) and double positive ($CD11^+/MHCII^+$) cell populations, obtained by flow cytometry, were comparable to the results of cell differentiation obtained by microscopy after May-Grünwald-Giemsa (**MGG**) staining by analyzing 165 raw milk samples from quarter milk of healthy and diseased cows with both methods. Immunofluorescence was performed with Anti-CD11b and Anti-MHCII antibodies on isolated PMN, cultured bovine macrophages, and milk samples to further confirm the method and antibody stainings.

MATERIALS AND METHODS

Milk from cows was taken during the daily milking, so this analysis did not require approval by an Institutional Animal Care and Use Committee or Institutional Review Board.

Chemicals and Reagents

All chemicals were purchased from Merck, except Walloxene (110/80, Wall Chemie), Sheat Fluid, Cytoflex daily quality control (QC) fluorospheres (Beckman Coulter), and Hoechst 33342 (Thermo Scientific). According to the manufacturer's instructions, PBS was prepared from tablets (P4417, Merck) and autoclaved, 5 tablets were added to 1 L of H_2O (PBS 1×), and 25 tablets were added for PBS 5× concentration.

Sample Collection and Preparation

For the SCC comparison (described in the section on validation of direct SCC) with ISO 13366-2 (ISO, 2006), 240 samples from individual cows belonging to one herd of different breeds were collected. For the dSCC comparison (described in the section on cell differentiation) with MGG staining, quarter milk samples from 165 healthy cows (belonging to one herd and collected by the producer) or diseased cows (collected by veterinarian after diagnosis of mastitis) were collected. All milk samples were collected under aseptic conditions in 50-mL Falcon tubes and immediately stored at 4°C. Samples were analyzed no later than 48 h after sample collection. No preservatives were added for the 2 comparison trials; however, proficiency testing was performed with preserved (bronopol) standard samples (used in the validation study). No influence of preservatives was observed on the results of the flow cytometry results; analysis was still possible if the samples were supplemented with bronopol or acidiol before arriving in the laboratory (e.g., dairy herd improvement samples). Before analysis, milk samples were heated at 37°C for 15 min and then gently mixed by inverting the tubes. The heating and mixing steps were repeated 3 times in total (a total of 45 min of heating and mixing) to optimize the separation of cells from fat.

Cell Staining and Antibody Incubation

For SCC, cells were incubated with the cell-permeable DNA dye Hoechst 33342 (final concentration 2 μg/100 μL of milk) to analyze the total number of cells. For dSCC, the antibody incubations were directly performed in the milk samples without any washing or centrifugation before DNA staining. For cell differentiation, milk samples were incubated with fluorescein isothiocyanate (**FITC**) mouse anti-bovine CD11b (CC126, MA5–28274, Fisher Scientific) and Janelia Fluor 646 mouse anti-bovine MHC (class II; CVS20, NBP2–34848JF646, Novus Biologicals). Antibodies (antibody mix) were diluted 1:200 in PBS 1× containing 5% BSA (PBS-BSA, 5%). Both antibodies were shown to react specifically with bovine cells (Weiss et al., 2001). For all samples, 100 μL of antibody mix was added to 100 μL of milk and incubated at 37°C in the dark for 1 h (with gentle orbital shaking). The same method was performed on samples of isotype controls (FITC mouse IgG2b, negative control IgG2b; MCA691F, Bio-Rad) and Janelia Fluor 646 mouse anti-bovine CD26 (CC69, MCA1652A647, Bio-Rad; CD26 was used instead of a JF646 IgG1 control). For the IgG blocking experience, the samples were incubated at 37°C with a dilution

of IgG mix from mouse serum (I5381-5MG, Sigma) at a final concentration of 1 mg/mL in PBS-BSA 5% for 30 min before antibody incubation (Okino et al., 2019). Then, 300 μL of PBS-BSA 5% was added to each sample, and the samples were again incubated at 37°C in the dark for 15 min.

Immediately before the analysis, 2 μL of Hoechst 33342 (50 μg/mL stock) was added to an F-bottom standard 96-well plate, and then 400 μL of sample and 30 μL of clearing solution (**CL**) were added to the aforementioned plate. The maximum volume of a 96-well plate is 400 μL, and with a total of 432 μL, the well was slightly overfilled. However, it was carefully checked that no spillage or carryover occurred between samples. (If possible, the use of deep-well plates for larger volumes is recommended.)

Milk Clearing

The CL contained 11 g/L Triton X-114 (Sigma-Aldrich, X114–500ML), 6.5 g/L Walloxen ID110/80 (Wall Chemie GmbH 180725), 0.4 g/L ammonium chloride (Sigma-Aldrich, 213330–25G), and 0.1 g/L ammonium formate (Sigma-Aldrich, 156264–500G), and was prepared with deionized water. Just before analysis, a total of 30 μL of CL was added directly to the microplate per 100 μL of milk. If the milk was diluted (e.g., 1:2), the clearing volume was reduced (e.g., 15 μL) in relation to the milk volume. Analysis of the samples within 30 min of adding the CL was necessary, because cell integrity was affected by the CL after a longer incubation time.

Flow Cytometer

This method was developed on a Cytoflex (Beckman Coulter) equipped with 3 lasers (405, 488, and 638 nm), and it was tested on a MACSquant (Miltenyi) and a Canto II (BD) to show its applicability to other instruments. All 3 flow cytometers belonged to the so-called "adjustable, open systems," according to the International Dairy Federation (2021a). As a quality control for cytometer performance [see also instrument qualification as described in ISO (2018)], the injector volume was calibrated, and before each run, quality control with fluorescent beads was performed (Cytoflex QC fluorospheres, Beckman Coulter). Based on sample autofluorescence, the threshold (**TH**) was set in the violet 610-nm height (**Vio610-H**) channel (excitation 405 nm/emission 610 nm) by running a sample after clearing. Alternatively, TH could be set in the PB-450-H channel [excitation 405 nm/emission 488 nm, corresponding to 4′,6-diamidino-2-phenylindol (DAPI) or V1 on other flow cytometers].

Analysis of Total Cell Counts

Cell counting and differentiation can be performed in 2 separate runs at different sample dilutions to avoid any loss of precision due to sample dilution. For SCC, a total of 100 μL of milk sample, without dilution and prior antibody incubation, was pipetted to the Hoechst-containing microwell (final concentration 2 μg/mL) for precise cell quantification. Subsequently, 30 μL of CL was added to the sample. Each sample was measured in triplicate. After comparing forward scatter height (**FSC-H**, x-axis) and Pacific Blue 450-nm height (PB-450-H, *y*-axis) channels in a dot plot (excitation 405 nm/emission 450 nm, equivalent to DAPI, V1 on other flow cytometers), the cell population was separated from the cloud of MFG (P1). Once gated, the flow cytometer results in events per microliter in gate P1. The flow cytometer stopping rule was set at 15 μL of analyzed volume. The cell concentration in the milk was calculated as follows: average count (cells/ μL) × 1,320. The 1,320 factor is obtained by dilution (100 μL of sample + 30 μL of CL + 2 μL of Hoechst) × 1,000 (conversion factor cells/μL to cells/mL). Additional dilution factors need to be considered for samples above 1.2×10^6 cells/mL.

Quantification of dSCC

Polymorphonuclear leukocytes and APC populations were separated, and cells were quantified after antibody incubations. Flow cytometry stopping rules were set to 10,000 events counted in the gate P1. Events counted in this gate were further separated on a second dot blot with FITC (CD11b) and JF646 (MHCII), represented on the *x*- and *y*-axis, respectively. Polymorphonuclear leukocytes are $CD11b^+/MHCII^-$ and APC are double positive $CD11b^+/MHCII^+$.

MGG Staining and Cell Counting

For MGG staining, 400 μL of milk sample was diluted in 1,600 μL of PBS in a 2-mL tube, mixed by inversion, and centrifuged at 500 × *g* at room temperature (**RT**) for 10 min. The supernatant was carefully removed, and the cells were resuspended in 1,600 μL of PBS. Samples were again centrifuged at 500 × *g* at RT for 10 min, supernatant was removed, and 200 μL of PBS was added to the sample. Then, 10 μL of sample was pipetted and evenly spread (using a glass slide) onto superfrost glass slides (AAAA000083##32E, Thermo Scientific) and dried at RT. The glass slides were defatted with xylol for 5 min and fixed with 96% ethanol for 5 min. Extra liquid was removed with the help of a paper, and the sample was dried at RT.

Samples were incubated with MGG eosin methylene blue solution (1.01424.1000, Merck) for 5 min and briefly rinsed in H_2O before incubation with a 1:20 diluted Giemsa azur eosin methylene blue solution (1.09204.1000, Merck) in H_2O for 25 min. The slides were rinsed with H_2O and dried.

A total of 100 cells per sample were counted and optically assigned to 3 cell types: PMN, macrophages, and lymphocytes.

Macrophage Cell Culture

The bovine macrophage cell line was obtained from C. J. Czurprynski (Woo et al., 2006). The cells were stored in liquid nitrogen (gas phase). The vial was thawed at 37°C in a water bath (fast). The content was then transferred in 10 mL of prewarmed (37°C) complete medium (RPMI1640 supplemented with 10% FCS, 100 IU/mL penicillin, and 100 μg/mL streptomycin). The cells were centrifuged at RT, at 300 × g for 5 min, and the supernatant was discarded. The cells were resuspended in a 5-mL complete medium in a 25-mL culture flask. Cells were incubated at 37°C with 5% CO_2 in a culture medium until they reached 70–90% confluency. Then, they were diluted 1:10 (cells:medium) with fresh medium in a 75-mL culture flask every second day or when confluent once the culture was stable. The culture was restarted from the cryostock when passage number 25 was reached.

Isolation of Bovine PMN Cells

Blood from bulls was well mixed with 10% anticoagulant solution (13.2 mmol/L Na_2KPO_4, pH 6.8, 15 mg/L EDTA, 7 mg/L NaCl), filtered through a tissue cloth to remove impurities, and cooled overnight at 4°C. The blood was centrifuged at 1,800 × *g* at 7°C for 30 min. The transparent serum and buffy coat were removed by aspiration. For lysis of red blood cells, the volume of the lower phase was measured and shaken in a glass bottle with 1.17 × the blood volume of hypotonic sterile EDTA solution (7.45 g/L) for 2 min. After that, isotonic conditions were re-established by adding 0.294 × the volume of the original blood with sterile 5 × concentrated PBS (PBS 5×). The blood was centrifuged again at 1,800 × *g* at 7°C for 20 min, and the cell pellet was resuspended with 1 × PBS (0.5–1 mL per 100 mL of cell suspension). Two more lysis steps were performed: addition of 6 × the volume of the cell suspension of EDTA solution, followed by 2 min shaking, then stopping the lysis with 1.5 × of the cell suspension volume with PBS 5 × . Between each lysis step, the suspension was centrifuged at 1,800 × *g* at 7°C for 20 min, the supernatant was discarded, and the pellet was resuspended in PBS 1 × (0.5–1 mL per 100 mL of cell suspension). The suspension was again washed twice with PBS 1 × by resuspending the pellet in PBS 1 × and subsequent centrifugation (1,800 × *g* at 7°C for 20 min). The final white pellet was resuspended in a fixation solution (UHT-treated skim milk, ethanol 20%, bronopol 0.05%, polyethylene glycol 0.1%).

Immunofluorescence

According to the manufacturer's recommendations, the gold glass slides were coated with Poly-L-Ornithin (Sigma-Aldrich, P4957, 50 mL) 1 d before the analysis. Cells were obtained by extraction from milk, and 400 μL of milk samples was pipetted in a 1.5-μL reaction tube with Safe-Lock cap (Huberlab). A total of 1,000 μL of 1 × PBS was added to each sample. Samples were heated at 37°C for 15 min and then gently mixed by tube inversion. The samples were centrifuged at 400 × *g* for 10 min at room temperature. The supernatants were discarded, and the pellets were resuspended in 1,000 μL of 1 × PBS and transferred to new tubes. This centrifugation step was repeated twice. The obtained cells were resuspended, and the cell suspension (20 μL) was pipetted onto the coated slides and incubated at RT for 2 h. The cells were fixed using 100 μL of PBS containing 4% paraformaldehyde (PBS-PFA 4%) at 4°C for 1 h.

Nonspecific binding sites were saturated with PBS-BSA 5% at RT for 1 h. Primary antibodies mouse anti-bovine CD11b (Clone MM10 A, WS0600B-100, Kingfisher Biotech) and mouse anti-bovine MHCII (Clone CC108, MCA5656) were diluted in a ratio of 1:200 in PBS-BSA 5% before overnight incubation at 4°C. The samples were washed 3 times with PBS containing 0.01% Tween 20 (T-PBS). Secondary antibodies FITC rat anti-mouse IgG2b (55395, BD, CH) and allophycocyanin rat anti-mouse IgG1 (550874, BD) were diluted in a ratio of 1:100 in PBS-BSA 5%, and they were incubated in the dark for 2 h. Nuclear stain Hoechst (final concentration 2 μg/mL) was added and incubated at RT for 5 min. Samples were washed 3 times with T-PBS 0.01% and analyzed under a microscope (Leica DM2000). Pictures were taken at a magnification of 1,000× using a Leica MC190 HD camera.

Statistical Analysis

The method comparisons between the new SCC and the routine analysis with ISO 13366-2 and the new dSCC and MGG staining were evaluated by using a Bland-Altman plot (Bland and Altman, 1986).

The evaluation of the proficiency testing was performed with Systat 13 software, R version 4.1.0 (R

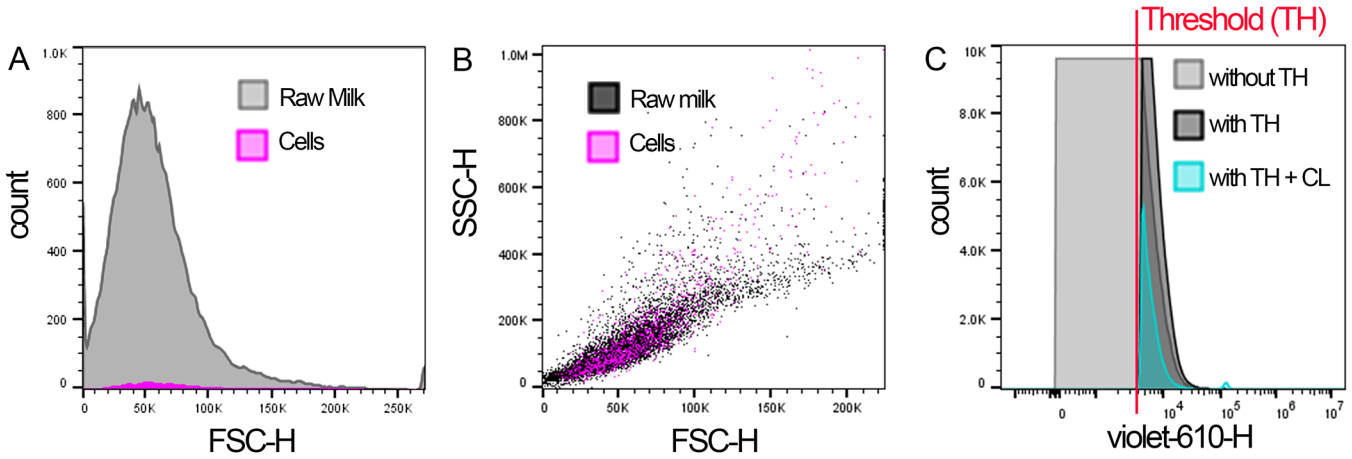


Figure 1. Gating strategy for total cell count. The histogram of raw milk without threshold setting (gray) and somatic cells (backgated in purple) representing forward scatter height (FSC-H; particle size) on the x-axis and cell counts on the y-axis (A); dot plot of the same sample showing FSC-H on the x-axis versus side scatter height (SSC-H; surface roughness) on the y-axis (B); histogram overlay of raw milk with 3 different settings: without threshold setting (without TH, gray), with threshold (TH) setting (red line) in the violet 610-nm height channel (with TH, dark gray), and with the addition of CL (with TH + CL, green; C).

Core Team, 2021), and Package "outliers" version 0.14. In total, proficiency testing was performed in 6 different laboratories. The mean values of the laboratories and operators were (robust) trimmed; the consensus values calculated from the means of these trimmed values were established based on ISO 5725-5 (ISO, 1998), Algorithm A. Cochran outliers were excluded. Z-values were calculated from the robust consensus values and the interpolated population standard deviations R based on the ISO 13366-2:2006 standard (ISO, 2006). Values within 2 standard deviations, corresponding to Z-values between −2 and 2, of the mean value were considered acceptable.

RESULTS

Challenges of Somatic Cell Counting in Milk

The main challenge of somatic cell counting in milk with flow cytometry was the presence of high numbers of fat globules of the same size as the cells. Therefore, it was impossible to differentiate them from the background based on size (FSC-H) or cell granularity (side scatter height, SSC-H) without labeling (Figure 1A, 1B). In the Vio610-H autofluorescence channel, MFG have a slightly lower intensity than somatic cells; therefore, TH was set in this channel to exclude most MFG, resulting in a higher signal-to-noise ratio indicated in red on the dot plot (Figure 1C). Moreover, the cells in a raw milk sample from a healthy cow represented less than 0.005% of the total events in the P1 gate, as shown for raw milk with less than 40,000 cells/mL (Figure 2A). To illustrate the instrument setting, the raw milk sample was analyzed 3 times under different settings: (1) without TH in light gray, where no peak of cells was visible but only the bulk of MFG; (2) after setting the TH (Figure 2B, dark gray); and (3) in teal color after sample clearing (Figure 2C, 2D, 2E), cells appeared as a small peak separated from MFG in pink (Figure 2C, 2F).

Strategy for Total SCC Based on DNA Staining

As described in Figure 1, the precise quantification of total cells, even in samples with high numbers, is hampered by the fact that the cells represent less than 0.5% of the total events after setting a TH (Figure 2B). Therefore, milk samples were treated with CL, which reduced the MFG considerably in size and number to achieve better sensitivity. Clearing solution was added to the milk sample immediately after DNA staining with Hoechst (or differential cell counting after antibody incubation) without other sample treatments. Better separation of cells from the background and a considerable reduction in signal-to-noise ratio were obtained after adding CL, as shown by the histogram of raw milk samples with low counts after DNA staining (PB-450-H, *x*-axis; Figure 2C). The sensitivity was increased by a factor of approximately 500, with the cells forming a small peak highlighted in pink, as illustrated in Figure 2C and 2E (Hoechst^{+}). In addition, higher precision in cell counts could be achieved by setting the cell gate (P1) in a 2-dimensional dot plot, separating the cells based on size (FSC-H) on the *x*-axis and nuclear staining (PB-450-H) on the *y*-axis (Figure 2F). The effect of CL on the depletion or enrichment of cer-

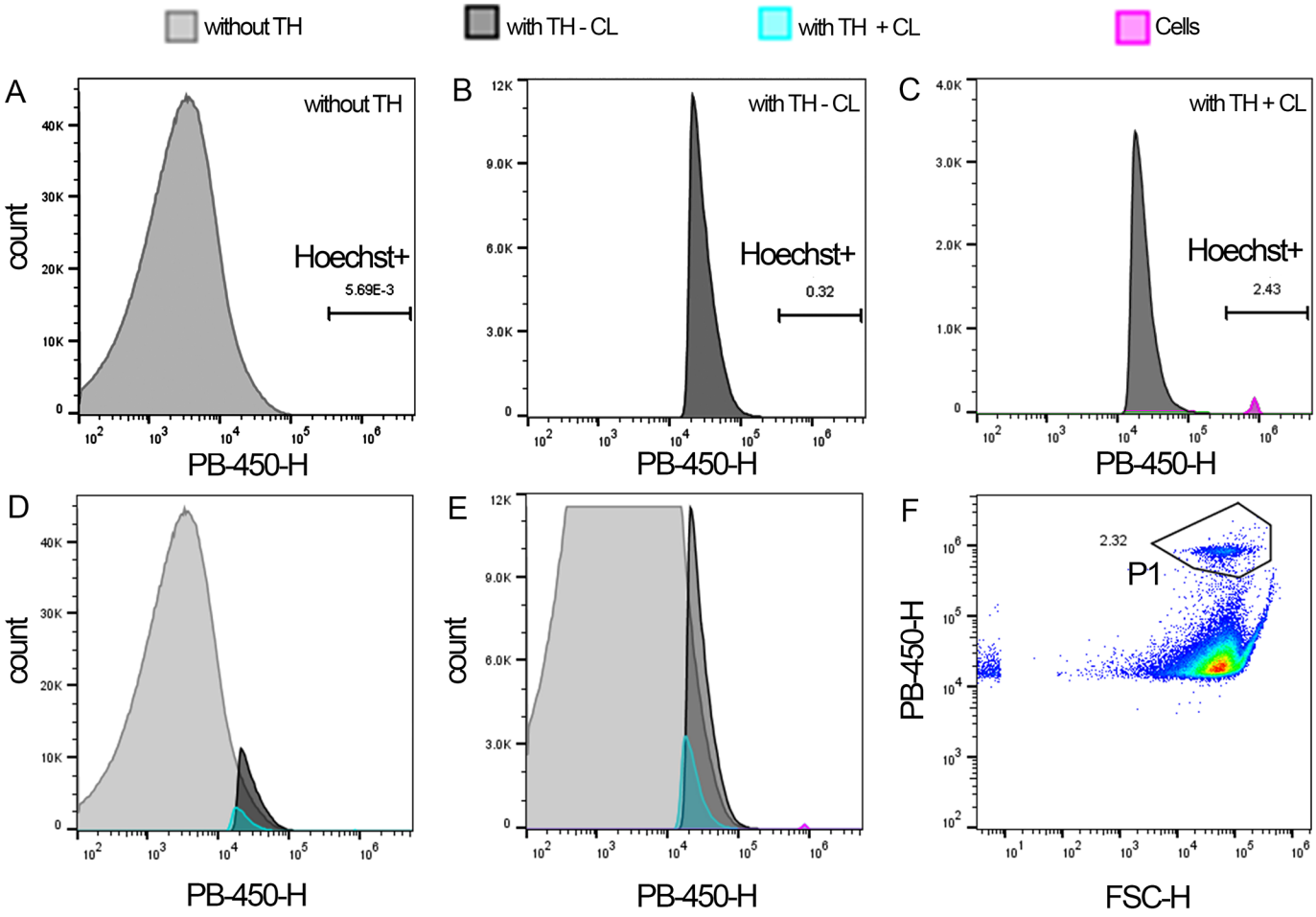


Figure 2. Noise reduction and cell-gating strategy for SCC. Histogram of raw milk without threshold (TH) setting showing Hoechst positive events (PB-450-H, Hoechst$^+$) on the x-axis, representing 0.005% of the total cells (without TH; A). The same sample with applied TH in the violet 610-nm height (autofluorescence) channel, with Hoechst positive (Hoechst$^+$) representing 0.32% of total cells (TH – CL; B). The same sample with additional clearing (CL), with 2.43% of Hoechst positive (Hoechst$^+$) of total cells (with TH + CL; C). Overlay of the 3 different settings shown in parts A–C (D), and the zoomed version of the same (E). Dot plot representation of raw milk, allowing a 2-dimensional separation of cells from noise, with forward scatter height (FSC-H) on the x-axis and Pacific Blue 450-nm height (PB-450-H) on the y-axis, and the color indicates an increase in the frequency of events from blue to red. Cells are gated in P1, representing 2.32% of the total events (F).

tain cell populations in dSCC was within the variability of the method if the clearing occurred no longer than 30 min before the analysis (Supplemental Figure S1 and Supplemental Table S1; https://doi.org/10.6084/m9.figshare.21070297; Egger et al., 2022).

Although the cells were separated at a specific peak on the histogram (Figure 2E), the quantification using the dot plot was more precise due to the overlap of MFG with the cell population in the histogram (Figure 2F). This result was excluded from the gate on the 2-dimensional representation. Total cell counts were directly analyzed in the samples after DNA staining without the need for an additional instrument calibration with standards of previously defined cell numbers using the new method (direct counting method as described in ISO 20391-1:2018 (ISO, 2018). Therefore, the new flow cytometry method was named direct SCC to differentiate it from the existing ISO flow cytometry routine method 13366-2 (ISO, 2006), for which a previous calibration or an adjustment of slope and intercept is requested, based on 2 to 10 standard samples with assigned values.

Validation of Direct SCC and Comparability with ISO 13366-2

Precision numbers for direct SCC were calculated based on a triplicate analysis of 244 individual raw milk samples. An intralaboratory reproducibility (R_{intra}) of 5.5% (7.2×10^3 cells/mL) and a CV of 5.8% were obtained with this method for samples with cell counts of more than 10×10^4 cells/mL. These data were compa-

rable to the precision data of the ISO 13366-2 routine method for total cell counts, with values between 3 and 6% for cell counts of 1,500,000–150,000 cells/mL (ISO-13366-2, ISO, 2006). A method comparison between direct SCC and ISO 13366-2 was statistically evaluated using a Bland-Altman plot (Bland and Altman, 1986) with the same set of 244 samples. The average results of both methods (in Log_{10} transformed values) are shown on the Bland-Altman graphical representation on the *x*-axis and the differences between the 2 methods on the *y*-axis (ISO, 2006). The mean difference (bias) between the 2 methods was 2.6×10^3 cells/mL, and over 95% of all samples were within the upper and lower limits of agreement of the 2 standard deviations (Bland and Altman, 1986; Figure 3A). Furthermore, the new direct SCC was performed in the frame of proficiency testing between different laboratories. Five standard samples at various levels were analyzed in 7 other laboratories with either 8 (Figure 3B, 3E, 3F) or 11 individual instruments (Figure 3C, 3D). All other instruments performed the ISO 13366-2 routine method (ISO, 2006). The statistical evaluation detected no outliers and no deviation from linearity for all participating instruments and the precision data are summarized in Table 1. Moreover, the Z-values of the new direct SCC method were within the limits of ±2, indicating that the results were within ±2 standard deviations of the average from all laboratories. The 2 higher standards had a Z-value above 1.0, which could be improved by analyzing the higher dilution of these samples (Figure 3E, 3F). These comparability experiments showed that the SCC analyzed with the new direct SCC method was consistent with that of the ISO 13366-2 (ISO, 2006) routine method and the microscopic reference method ISO 13366-1 (ISO, 2008; data not shown). It must be noted that good comparability with the actual reference method ISO 13366-1 was most often achieved due to the poor repeatability of the latter (International Dairy Federation, 2021a). Method comparisons between more laboratories will be required for further method validation.

The new direct SCC method was tested on 3 different flow cytometers to confirm that it is independent of any specific equipment (Figure 3G). The results for 5 standard samples at different levels and a raw milk sample had good comparability and were close to the values obtained with the ISO 13366-2 routine method.

Direct Cell Differentiation and Quantification

Polymorphonuclear leukocytes and APC represent the main cell types that vary between milk from healthy cows to acutely diseased to chronically diseased cows and were targeted with specific antibodies for direct cell differentiation. Therefore, the samples were incubated with fluorescent antibodies against CD11b and MHCII. After antibody incubation, the permeable DNA staining dye Hoechst was added, and the samples were measured as described for total cell counting. The gating strategy was first performed for direct SCC by using a dot plot with FSC-H on the *x*-axis and PB-450-H on the *y*-axis, and considering the PB-450-H positive cloud appearing in the upper half of the graph (Figure 4, P1). An additional dot blot for cell differentiation, including the events in the gate (P1), was generated, representing the total number of cells. In this new dot plot, AP-H fluorescence was set on the *x*-axis and FITC fluorescence on the *y*-axis. Total cell counts were calculated from the events in the P1 gate in Figure 4A, 4C, and 4E, and differential cell counts were calculated based on the cell populations generated in Figure 4B, 4D, and 4F. Isotype controls performed for both antibodies showed very low interferences (<3%; Supplemental Figure S2 and Supplemental Table S2; https://doi.org/10.6084/m9.figshare.21070297; Egger et al., 2022).

In the upper left gate, $CD11b^+/MHCII^-$ events were defined as PMN and lymphocytes, double positive $CD11b^+/MHCII^+$, located in the upper right gate of the dot plot (or density plot), respectively, were defined as APC (e.g., macrophages; Figure 4B, 4D, 4F; Supplemental Figure S3, https://doi.org/10.6084/m9.figshare.21070297, Egger et al., 2022; density plot). Three representative samples from quarter milk samples with low SCC (<10,000 cells/mL; Figure 4A, 4B), high SCC ($>10^6 < 10^7$ cells/mL; Figure 4C, 4D), and very high SCC ($>10^7$ cells/mL; Figure 4E, 4F) are shown. With increased SCC, the relative abundance of $CD11b^+/MHCII^-$ cells increased from 85.9 to 95.65% and $CD11b^+/MHCII^+$ cells decreased from 13.5 to 4.05%.

To confirm the relative distribution of the 2 targeted cell populations of dSCC, 165 milk samples from quarters of healthy or diseased cows were analyzed in parallel by microscopy after MGG staining. For MGG staining, it was necessary to isolate the cells from the milk samples by centrifugation and washing. Therefore, to compare the methods, dSCC was performed exceptionally after the same sample preparation used for MGG staining. The cell population from dSCC (washed; $CD11b^+/MHCII^-$) was compared with the population of PMN and lymphocytes after MGG staining, yielding an average bias of 1.4% (Supplemental Figure S4A; https://doi.org/10.6084/m9.figshare.21070297; Egger et al., 2022), and the double positive ($CD11b^+/MHCII^+$) population was compared with the macrophages after MGG staining (average bias 1.9%; Supplemental Figure S4B).

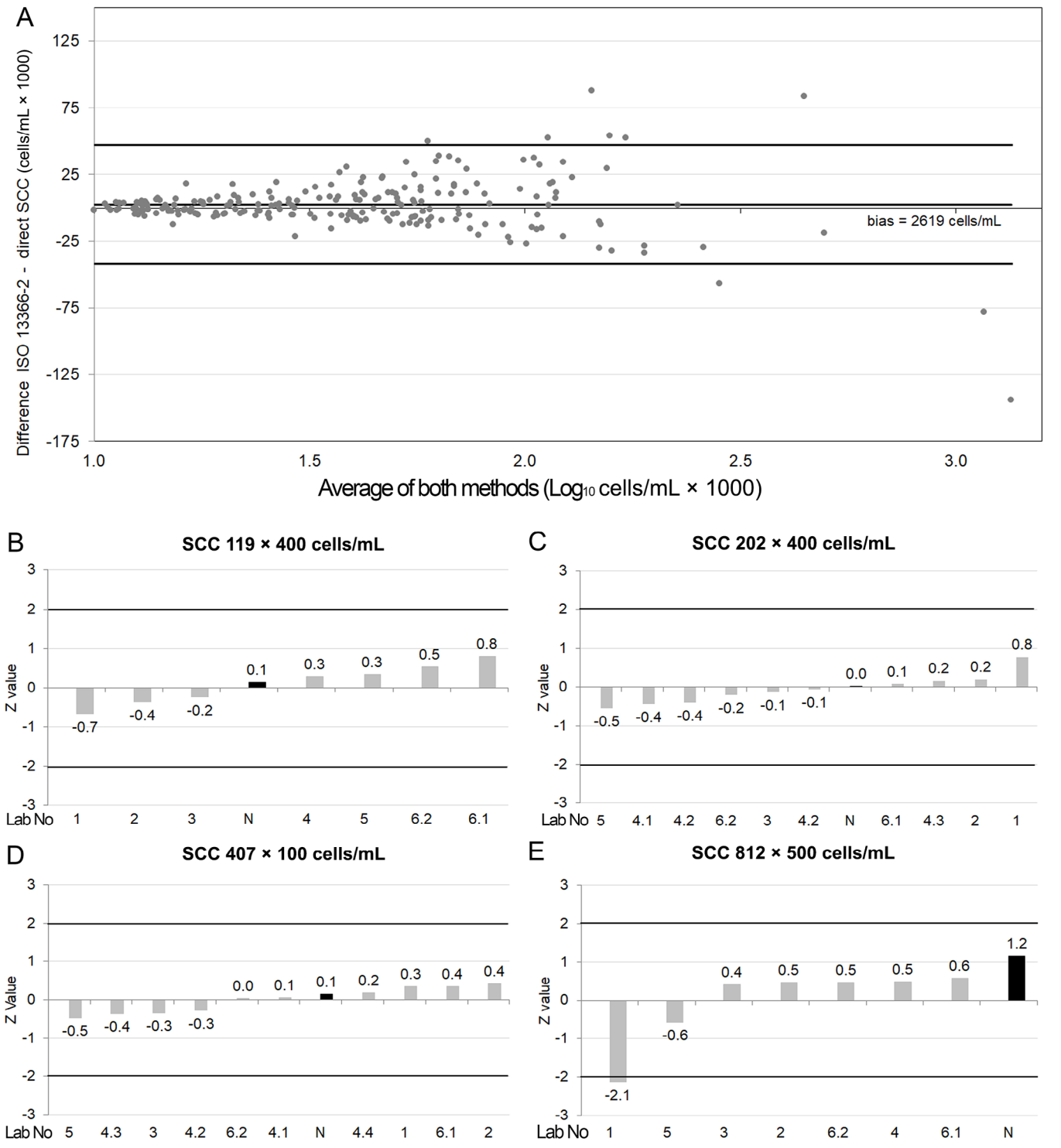
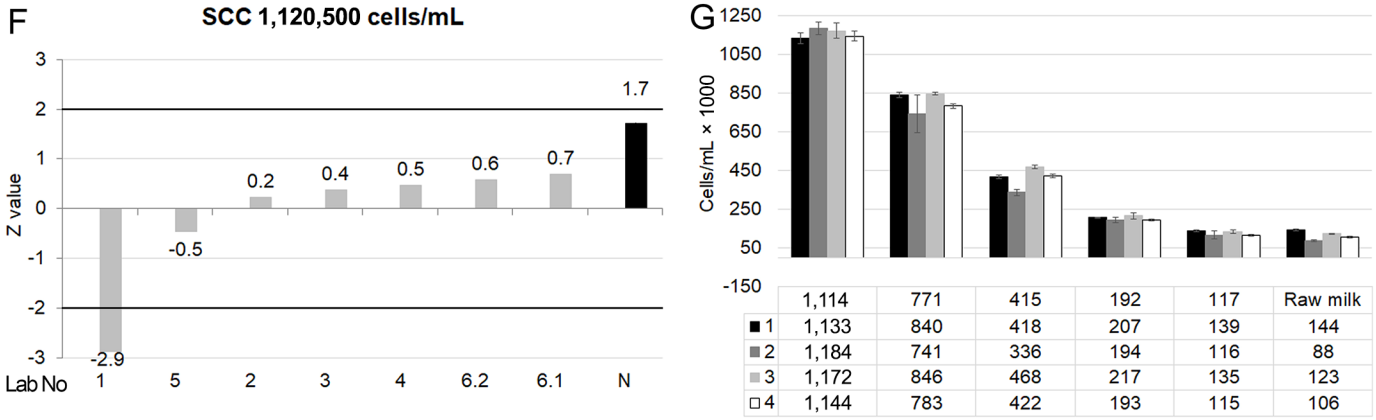


Figure 3. Method comparison between direct SCC and ISO 13366-2 (ISO, 2006). Bland–Altman method comparison between direct SCC and the ISO 13366-2 routine method (triplicates of $N = 244$). The average values of both methods in $\log_{10}$ scale are represented on the x-axis and the differences between the two methods are represented on the y-axis. The middle line represents the average difference (bias = 2,619 cells/mL, ISO 13366-2—direct SCC), and the upper and lower lines represent the limits of agreement of ±2 SD (A). The results were obtained by a proficiency test performed with the 5 levels of Agroscope SCC standards. Representation of the Z-values of different laboratories (1–6) participating with 1 or 2 instruments (e.g., 4.1–4.4) obtained for different levels of somatic cells, where *N* represents the new direct SCC method (B–F). Comparison of the new direct SCC method performed on 3 different instruments: Cytoflex (Beckman Coulter, black bars), MACSquant (Miltenyi, dark-gray bars), and Canto II (Becton Dickinson, light-gray bars), with the existing routine method ISO 13366-2, performed on a dedicated analyzer (Bentley SC; G).



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Verification of the dSCC Method by Fluorescence Microscopy

The dSCC method was verified by immunofluorescence of PMN isolated from the blood of bulls, as described in the previous section (Figure 5A–D), immortalized bovine macrophages (Woo et al., 2006; Figure 5E–H), and raw milk containing different cell types (Figure 5I–L). Cells were incubated with primary antibodies against CD11b (FITC labeled secondary antibody, green) as markers for PMN and lymphocytes, and secondary antibodies against MHCII (AP labeled secondary antibody, red) were expressed on APC only, for example, macrophages and monocytes. As expected, all PMN were clearly positive for CD11b and negative for MHCII (Figure 5B, 5C). In contrast, all bovine macrophages were double positive (Figure 5F, 5G). Around 10 to 15% of the cells were double positive, and most of the cells were positive for CD11b in the raw milk sample. The results obtained with the differential analysis performed by flow cytometry were confirmed by these qualitative results.

DISCUSSION

The routine analysis of direct SCC in milk using dedicated flow cytometers according to ISO 13366-2 (ISO, 2006) is based on calibration with certified reference standards (International Dairy Federation, 2021b) to guarantee the equivalence of analytical results at an international level. In contrast, the newly developed direct SCC flow cytometry method does not include such calibration. Nevertheless, total cell counts are established based on particle size and the presence of DNA—2 main features of somatic cells in both methods. Therefore, the total cell numbers were expected to agree with the 2 methods. This equivalence was confirmed by a direct method comparison between the new direct SCC and ISO 13366-2 with over 240 raw milk samples and performing interlaboratory proficiency testing. The precision of the new method was as well comparable to ISO 13366-2, also in samples with low SCC, where at least >500 cells will be counted in the gate P1 in a sample with 50,000 cells/mL (Figure 2F). However, more samples need to be analyzed in interlaboratory trials to confirm the statistical equivalence of different flow cytometers and laboratories. These initial results indicate that the method is not instrument dependent and can easily be transferred to other laboratories and instruments. As a minimal setting, the instrument should be equipped with a laser at 405 nm to assess the Hoechst signal, a laser at 488 nm (FITC, or APC), and a precise injector, allowing the monitoring of the sample volume needed to calculate absolute cell numbers rather than relative cell populations.

In addition to total cell counts, dSCC in milk is becoming more important in detecting and monitoring udder health status. The recently published high-throughput method based on DNA staining with acridine orange (Damm et al., 2017) is a significant step toward monitoring udder health at the routine level.

Table 1. Precision data obtained with the proficiency testing for total cell counts[1]

			Precision data proficiency testing										Precision data direct SCC			
Level	Labs	Inst	N	Mean	S_L	S_{rep}	S_R	RSD_L	RSD_{rep}	RSD_R	rep	R	R/rep	N	Mean	SD	RSD	rep
SCC 100	6	8	125	120.5	5.0	5.2	7.2	4.1	4.4	6.0	14.8	20.5	1.4	15	121.0	3.8	3.2	10.8
SCC 200	20	25	354	208.1	11.3	11.0	15.8	5.4	5.3	7.6	31.3	44.6	1.4	15	202.8	4.8	2.4	13.6
SCC 400	20	25	353	421.1	30.6	16.4	34.7	7.3	3.9	8.2	46.5	98.2	2.1	15	411.3	10.1	2.5	28.6
SCC 700	6	8	125	825.0	48.7	16.2	51.3	5.9	2.0	6.2	45.9	145.3	3.2	15	868.7	16.5	1.9	46.7
SCC 1,000	6	8	125	1,150.6	89.0	27.9	93.2	7.7	2.4	8.1	78.8	263.8	3.4	15	1,246.6	38.2	3.1	107.9

[1]Results are cell numbers per mL of milk $\times 10^3$. Samples at 5 different levels were analyzed. Levels 100, 700, and 1,000 were analyzed by 6 different laboratories (Labs) and 8 instruments (Inst), and levels 200 and 400 were analyzed by 20 different laboratories and 25 instruments, respectively. N = number of individual determinations; S_L = laboratory repeatability standard deviation; S_{rep} = repeatability standard deviation: S_R = repeatability standard reproducibility; RSD_L = relative standard deviation between laboratories; RSD_{rep} = relative standard deviation of repeatability; RSD_R = relative standard deviation of reproducibility; rep = repeatability; R = reproducibility; RSD = relative standard deviation.

In this flow cytometry-based method, in addition to SCC, a mastitis index based on the differential staining of certain cell types with acridine orange, without specific markers for different cell types, is given. Another automated system based on acridine orange staining, although not suited for high-throughput analysis, has been used in the study by Gonçalves et al. (2017) that investigated subclinical mastitis. This microscopy-based system has the advantage of measuring the absolute numbers of PMN, lymphocytes, and macrophages. In recent publications, these new methods were used to study the relation between SCC and dSCC, confirming that dSCC monitoring will be beneficial to improve animal health in the future and indicating that more research is needed until its implementation (Gonçalves et al., 2017; Zecconi et al., 2019; Bobbo et al., 2020).

Several other antibody-based flow cytometry methods for cell differentiation have been applied in the past; however, to our knowledge, these methods for dSCC in milk using specific antibodies for cell differentiation have all been performed after isolating and washing the cells from the milk background (Dulin et al., 1982; Schwarz et al., 2011; Pilla et al., 2013). Although others have not found significant differences in the distribution of cell types after centrifugation (Dulin et al., 1982), it is well known that cells remain in the upper cream layer after centrifugation (Caplan et al., 2013), which could change the ratio of specific cell types. Some controversial results in cell-type distribution found in the literature, such as the high percentage of lymphocytes in milk with low SCC, could also be explained by sample preparation (Dosogne et al., 2003; Schwarz et al., 2011). However, this high percentage of lymphocytes was not observed by (Damm et al., 2017) using a direct method. Therefore, a direct method for cell quantification and specific cell differentiation without centrifugation and washing steps is of paramount interest for monitoring udder health. In the newly developed direct method, CL was used to improve the separation of cells from MFG based on size. Two method comparisons were performed to verify that the treatment itself neither altered the number of cells nor the distribution of certain cell types. First, SCC was in agreement with ISO 13366-2 (ISO, 2006), and second, the dSCC cell populations were confirmed by microscopy after MGG staining. It has to be mentioned that this second method comparison was performed with cells isolated from milk which could have altered the distribution of specific cell types.

Antibody-based methods are highly specific and can be adapted to other research questions or animal species. In this work, 2 central cell populations in milk were selected: PMN and lymphocytes together ($CD11b^+/MHCII^-$) and APC ($CD11b^+$, $MHCII^+$),

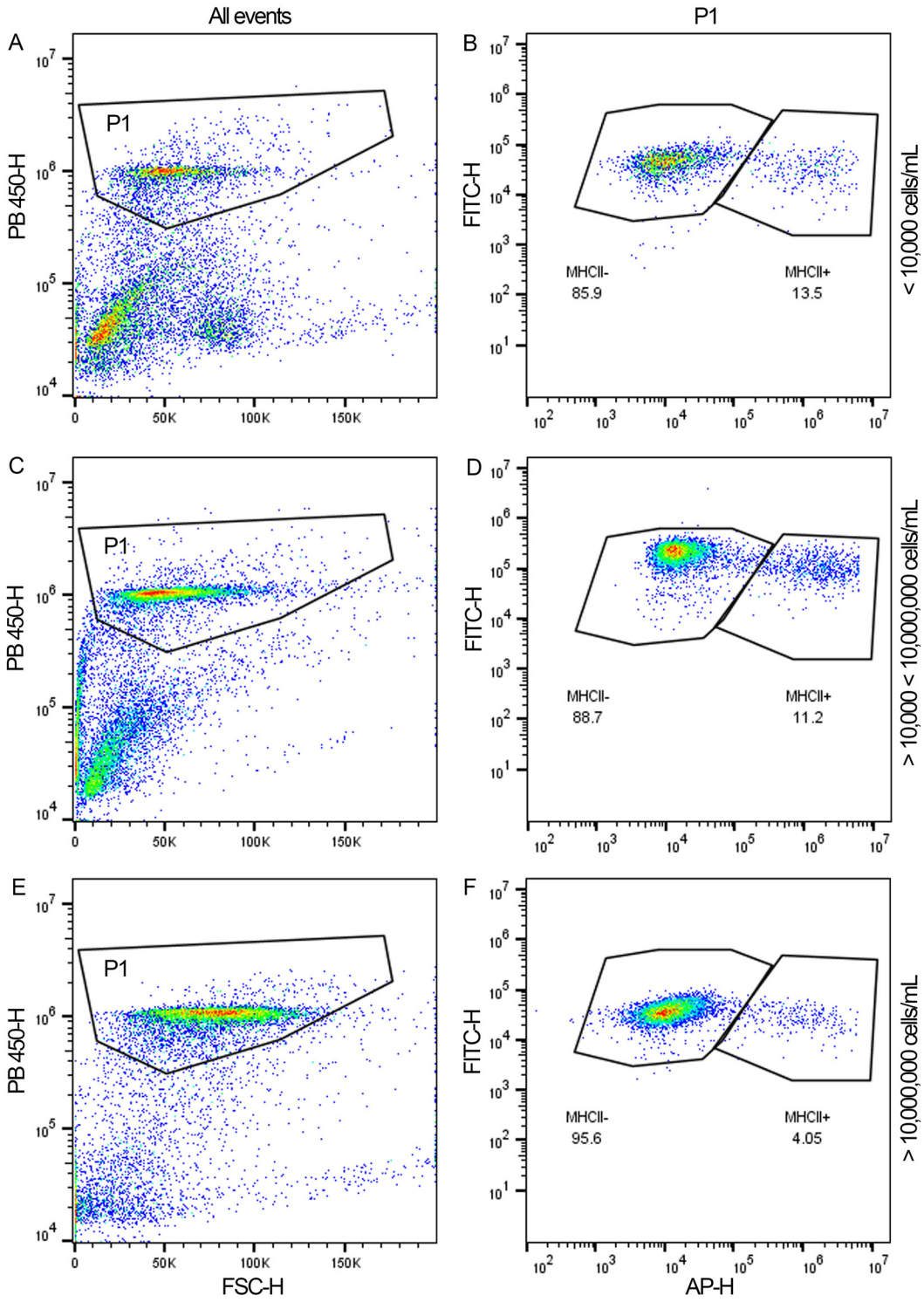

Figure 4. Cell differentiation using antibodies against integrin α-M (CD11b) and major histocompatibility complex class II (MHCII). Gating of total cells (P1) based on positive DNA staining in a dot plot with forward scatter height (FSC-H) on the *x*-axis and PB450-H on the *y*-axis (A, C, E). Dot plot for cell differentiation, selecting the events from gate P1 from the dot plot (A, C, E). The JF646-labeled MHCII antibodies are represented in the AP-H channel on the x-axis and the fluorescein isothiocyanate (FITC)-labeled CD11b antibodies on the FITC-H channel are represented on the y-axis, respectively. The distinction of 2 cell populations, $CD11b^{+}/MHCII^{-}$ events in the upper left gate ($MHCII^{-}$) and $CD11b^{+}/MHCII^{+}$ events in the upper right gate ($MHCII^{+}$; B, D, F) were performed. Representative quarter milk samples with low SCC (<10,000 cells/mL; A, B), high (>10,000 < 10,000,000 cells/mL; C, D), and very high (>10,000,000 cells/mL; E, F) are shown.

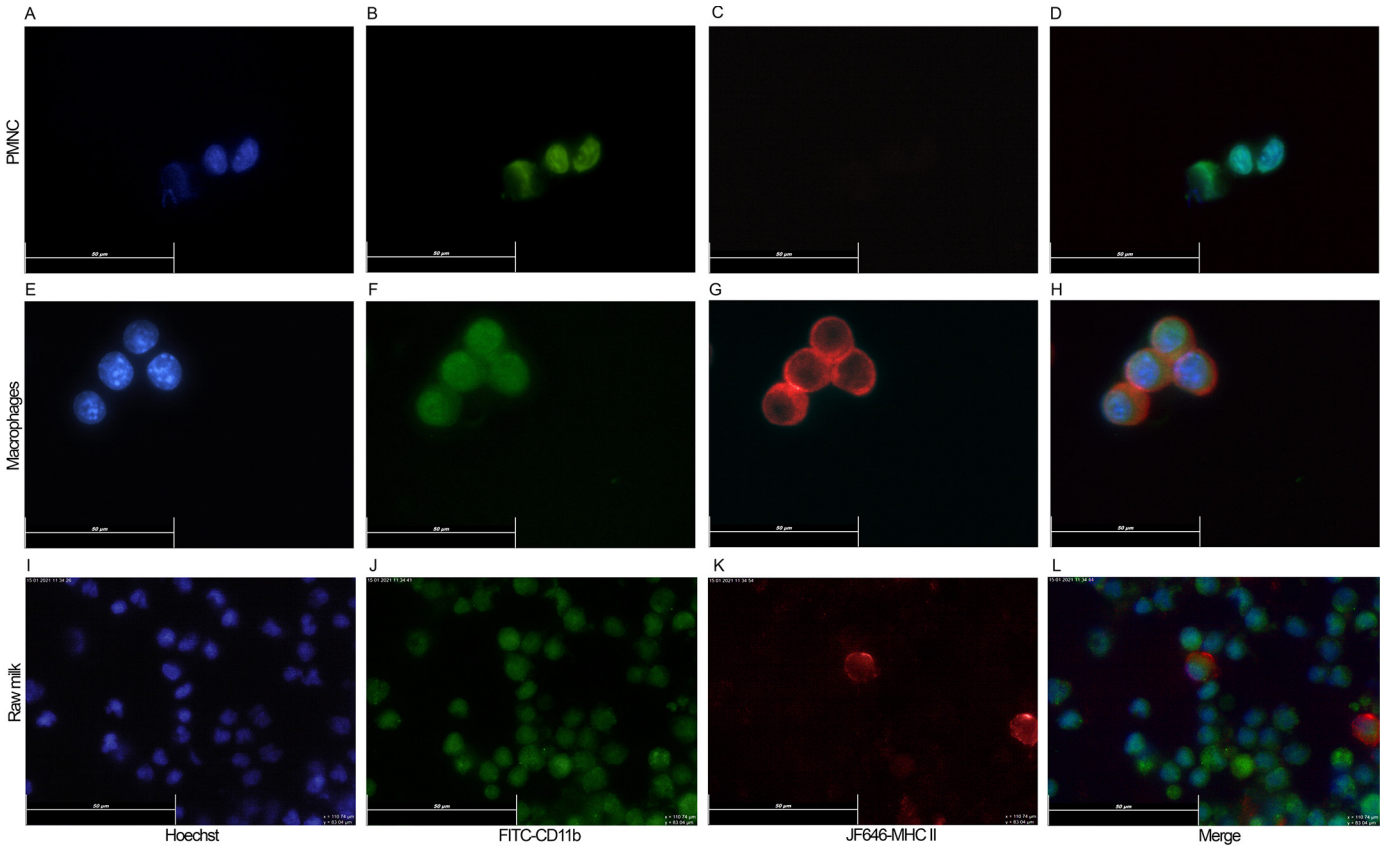

Figure 5. Verification of the differential somatic cell count (dsCC) method by fluorescence microscopy. Immunofluorescence staining of polymorphonuclear cells (PMNC) cells isolated from bull blood (upper lane), bovine macrophages (Woo et al., 2006; middle lane), and raw milk (lower lane), incubated with integrin α-M (CD11b; secondary antibody FITC labeled, green; B, F, J) and major histocompatibility complex class II (MHCII; secondary antibody APC labeled, red; C, G, K) antibodies, together with the nuclear counterstained Hoechst (A, D, I) and the merged pictures of all 3 channels (panels D, H, L).

because they are highly variable during mastitis (Schwarz et al., 2011; Pilla et al., 2013; Damm et al., 2017). It is known that antibody-based methods are limited by the availability of antibodies, are time consuming, and need trained technicians, resulting in higher costs compared with high-throughput methods. However, in the future, both types of methods will be required for improved dairy herd monitoring, automated methods with high throughput for milk producers or dairy plants, and specific methods that allow verification of standards, for quality assurance purposes, or in research.

CONCLUSIONS

The newly developed flow cytometry-based method based on DNA staining and specific antibody labeling without following the steps of centrifugation and washing allows for the direct analysis of SCC and dSCC in milk obtained from cows. The SCC and dSCC are consistent with the results from ISO 13366-2, and cell populations correspond to MGG results obtained by microscopy. This method can easily be adapted for other cell types or even milk from other species by selecting appropriate antibodies.

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