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# ANTOINETTE

## DN**A** a**n**d An**t**ib**o**dy Sta**in**ed Total and Diff**e**ren**t**ial Soma**t**ic C**e**ll Counting in Milk using Flow Cytometry

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*Agroscope, Switzerland*

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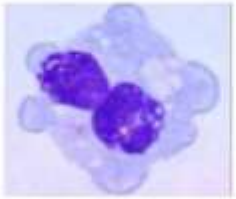
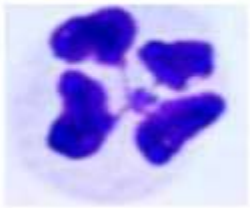
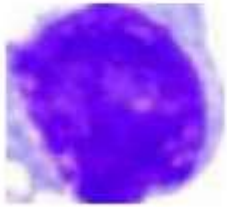
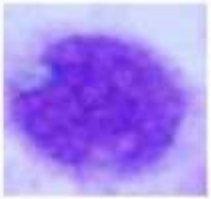
# Somatic Cells in Milk

| | Macrophages | PMNs | Lymphocytes | Epithelial cells |
|---|---|---|---|---|
| Visual aspect of stained somatic cells from cows |  |  |  |  |
| Morphological characteristics | 8-30µm<br>Many different forms of nucleus<br>Cytoplasm 0.5 to 10 x bigger than nucleus | 10-14µm<br>Intensively stained lobulated nucleus<br>Small cytoplasm, dense granules | 5-10µm<br>Intensively stained round nucleus<br>Very little cytoplasm | 10-14µm<br>Round nucleus<br>Cytoplasm weakly stained |

| **SCC** | | (%) | (%) | (%) | (%) |
|---|---|---|---|---|---|
| healthy milk | < 100'000/ml | 58 | 12 | 28 | 2 |
| mastitis milk | 100-400'000/ml | 25 | 63 | 11 | 1 |
| | > 400'000/ml | 3 | 87 | 9 | 1 |

*References*
*EN ISO 13366-1/IDF 148-1*
*Anke Schröder, Institute for Food Quality and Safety, University of Veterinary Medicine Hannover, Foundation*
*Petra Winter, AGES, Institut für Veterinärmedizinische Untersuchungen, Robert Koch Gasse 17, A-2340 MÖDLING*



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# Why Flow Cytometry?

- Thousands of cells can be analyzed in a standardized manner - high degree of accuracy and precision
- Possibility for protocol standardization independent of a specific instrument manufacturer and operator
- Opportunity to detect different cell populations in one run
- Routinely applied in the milk sector for quantification of total cells, over 2 Billions of analyses in milk per year
- Worldwide method of choice in clinical blood cell counting

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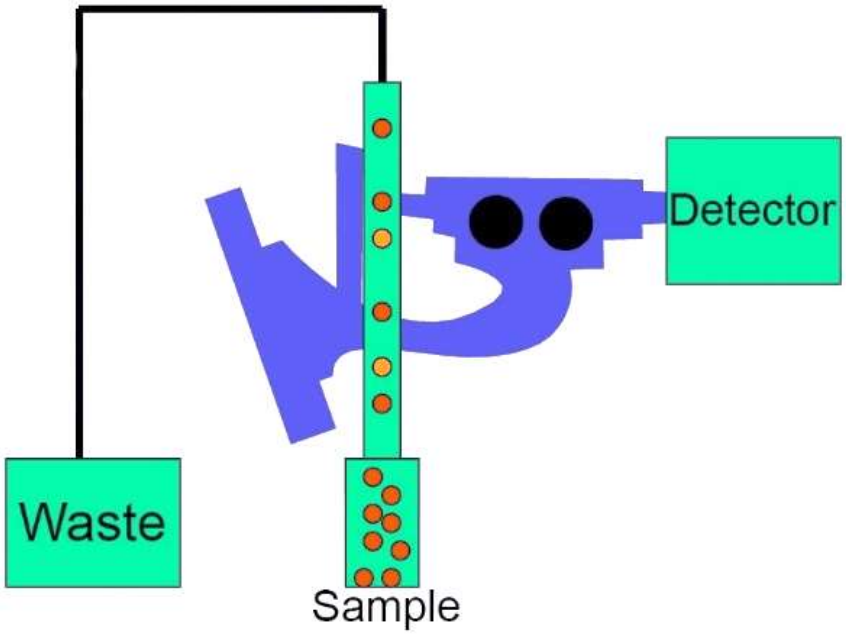
# Method principle I




Principle:
Isolated Particles in solution pass a detector and are counted and analyzed for the presence of specific markers.
A laser serves as light source



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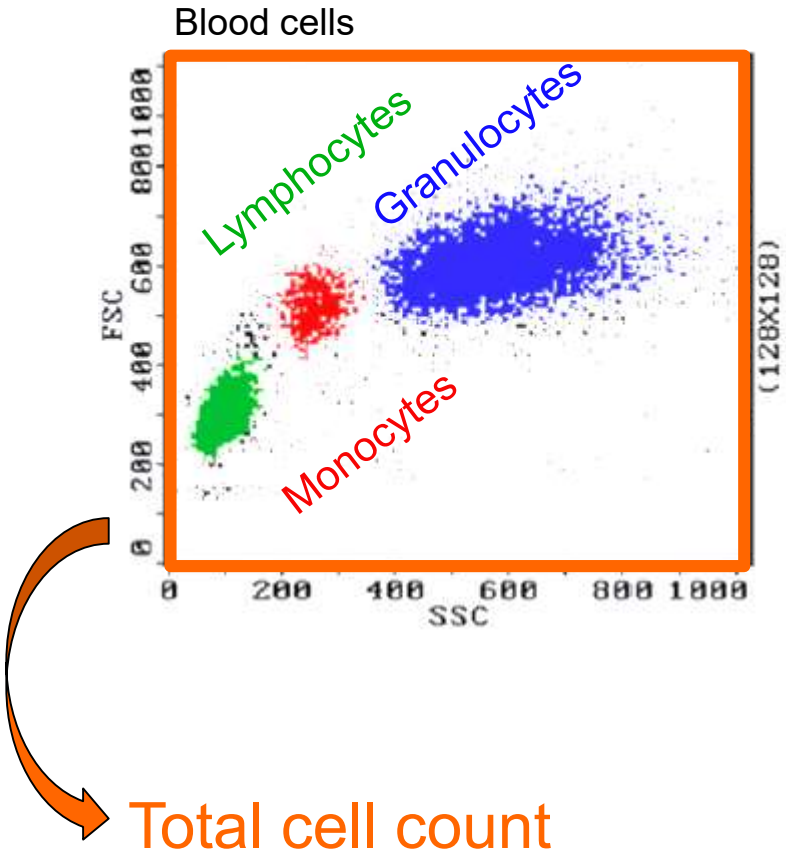
# Method principle II




Total cell count

Differentiation of cell populations possible, based on morphology using light scattering:

- Relative size
  → Forward Scatter (FSC)

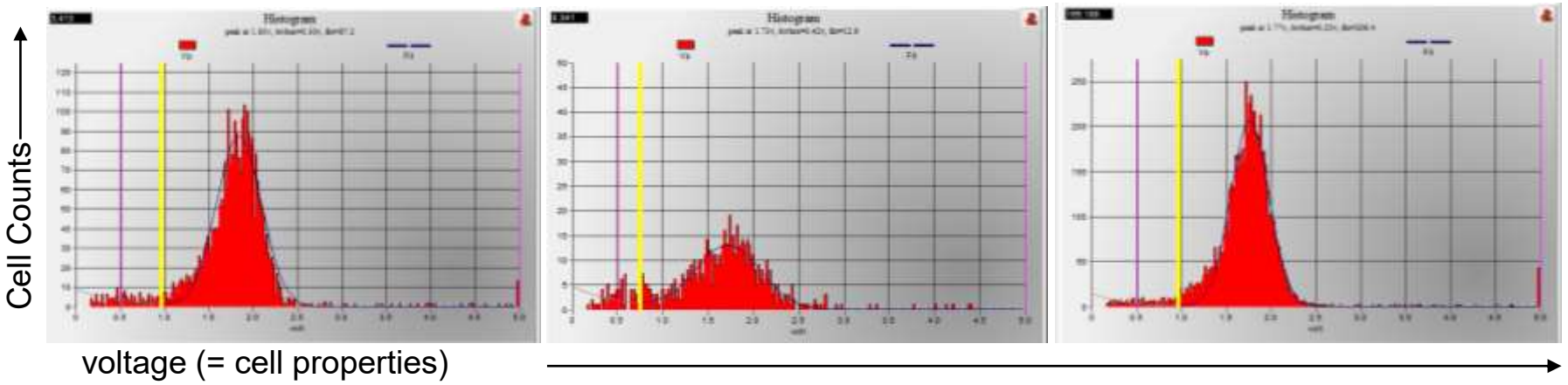
- Granularity or Complexity
  → Side Scatter (SSC)

Additional differentiation of cell populations based on specifically expressed proteins

- with fluorescently labelled antibodies

# Applications I: Total Cell Counts

- Routine analysis of total SCC according to ISO 13366-2 (based on DNA staining as the reference method 13366-1)



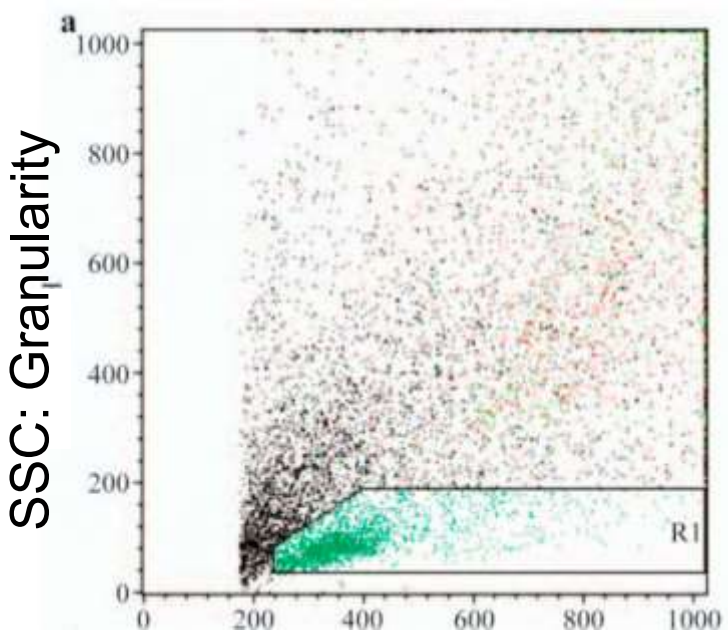

Standard
400’000 cells/ml

Raw milk, low
50’000 cells/ml

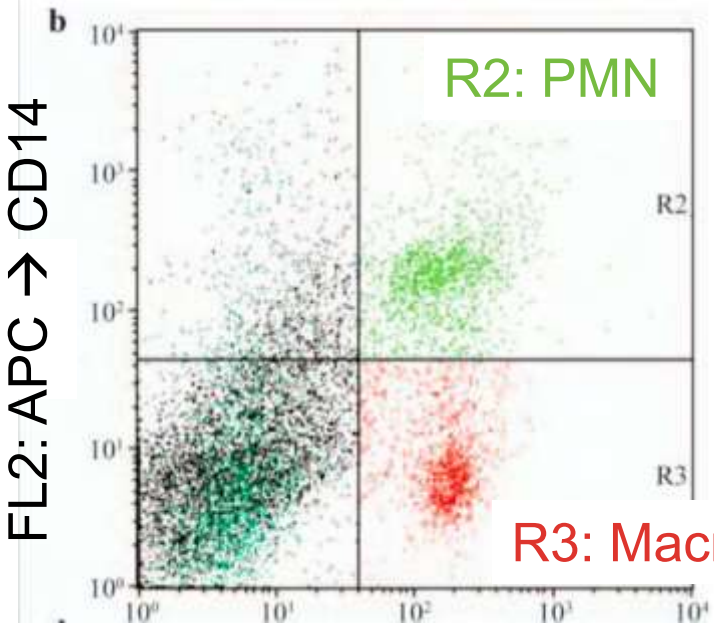
Raw milk, mastitis
500’000 cells/ml

# Applications II: Differential Cell Counting

- Analysis of Lymphocytes according to cell size and granularity
- Differentiation of PMNs from Macrophages using specific antibodies
- Relative distribution of cell populations




R1: Lymphocytes




Schwarz et al., J. Dairy Sci. 94:50335044, 2011

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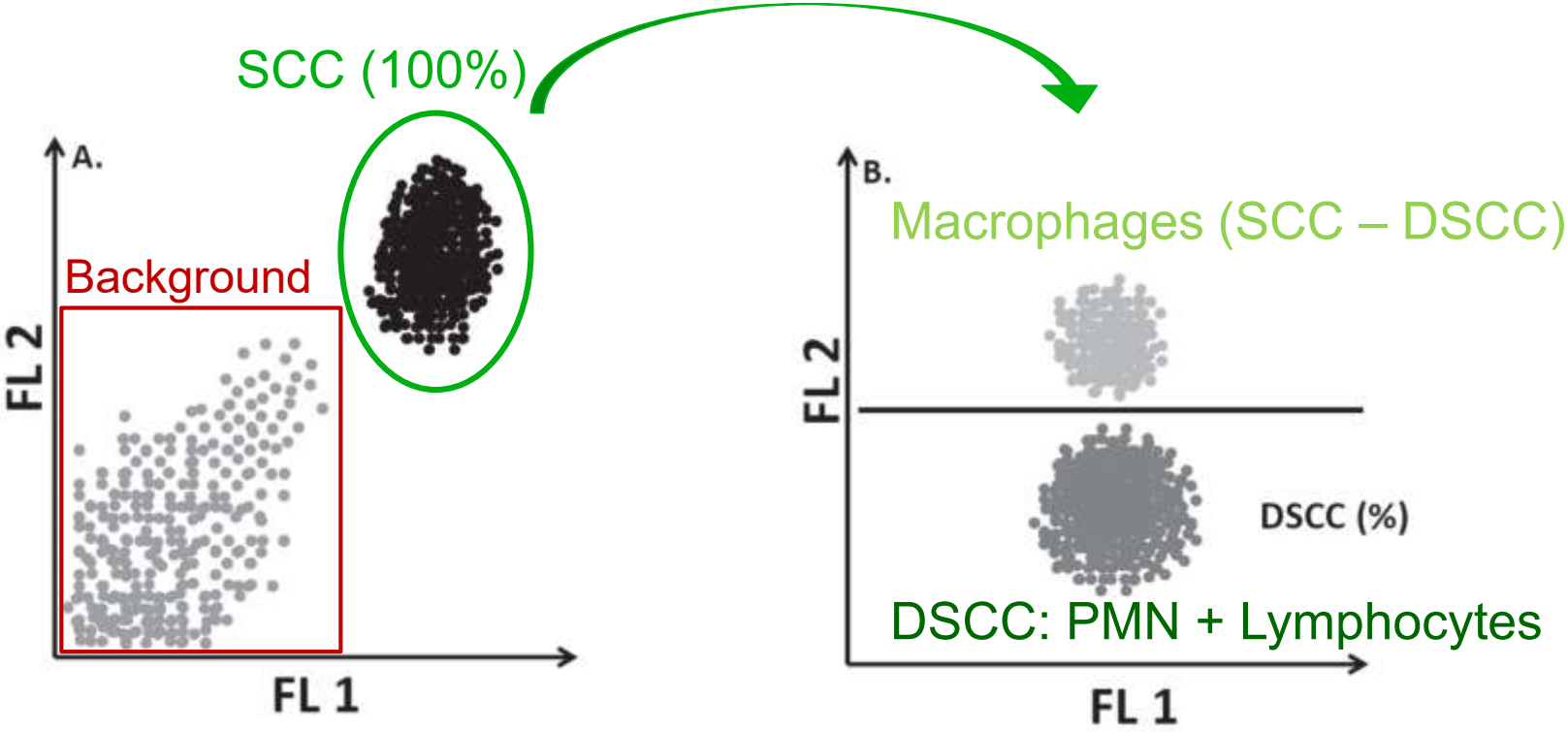
# Applications III: Routine Differential Cell Counting in milk

- Analysis of total cell counts using DNA staining
- Differentiation of PMNs + Lymphocytes from Macrophages in SCC subpopulation based on differences in FL2 staining → indication on mastitis status




Damm et al., J. Dairy Sci. 100:4926-4940, 2016

# Summary Applications of Flow Cytometry in milk:

- Flow cytometry methods based on DNA staining do not discriminate different cell types but give quantification results of total cells with a high repeatability and reproducibility

- Differential cell counting methods with specific antibodies used so far, needed centrifugation steps and represented therefore only relative populations of cells

- Routine DSCC (Foss) performed in one run is based on DNA staining and results in quantification of total cell counts and the differentiation of macrophages based on the same DNA staining. From this number, the PMNs are deduced, giving an indication on the mastitis status.

# Aim of the ANTOINETTE project:

Development of a flow cytometry method for the simultaneous quantification of total somatic cells and the individual quantification of immune cells involved in mastitis.

## Main Challenges:

1. Cells represent only one microscopic structure in milk → Casein micelles and milk fat globules are similar in size as the cells

2. Staining and immune detection needs to be done in situ to allow the precise quantification of cells and subsets of cells in the milk matrix → no centrifugation or washing steps that lead to cell loss



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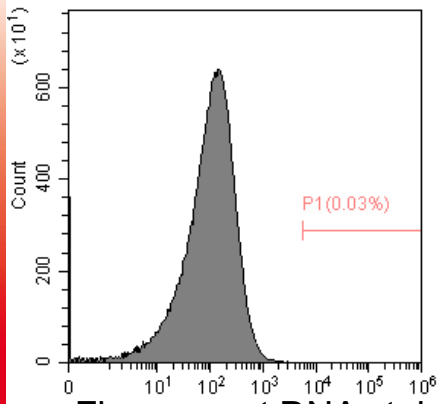
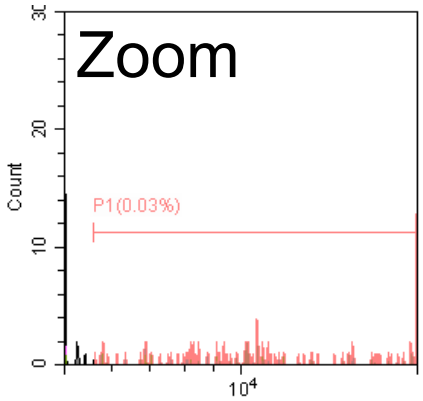
# Challenges:

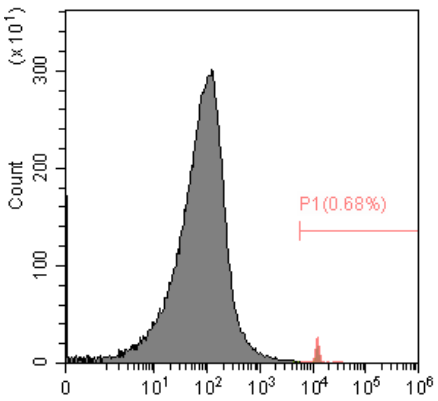
Pilote: 78500 cells/ml

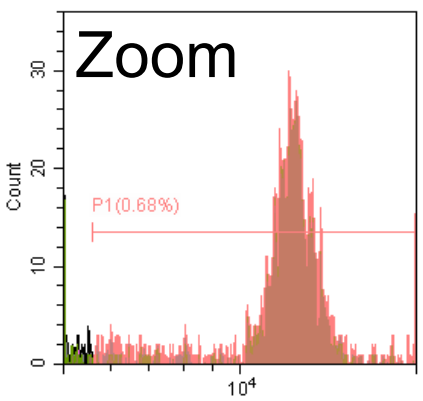


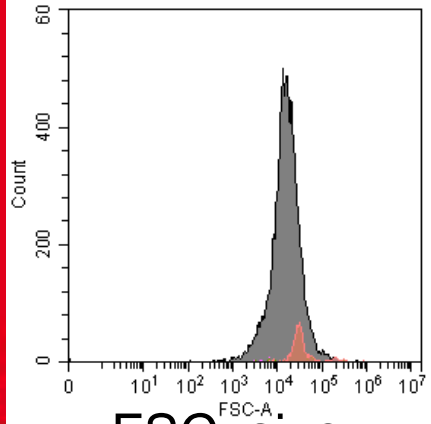

Fluorescent DNA staining

Standard: 807000 cells/ml

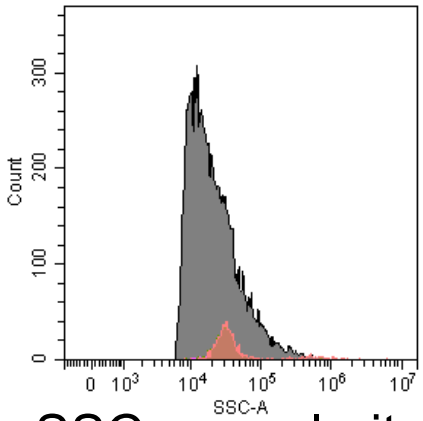



**Sensitivity and selectivity:** ratio of signal/ background is 1: 30'000 in a raw milk from a healthy cow




FSC: size




SSC: granularity

**Discrimination of cells from milk matrix** (casein micelles and milk fat globules) is not possible based on particle size (FSC) and granularity (SSC)



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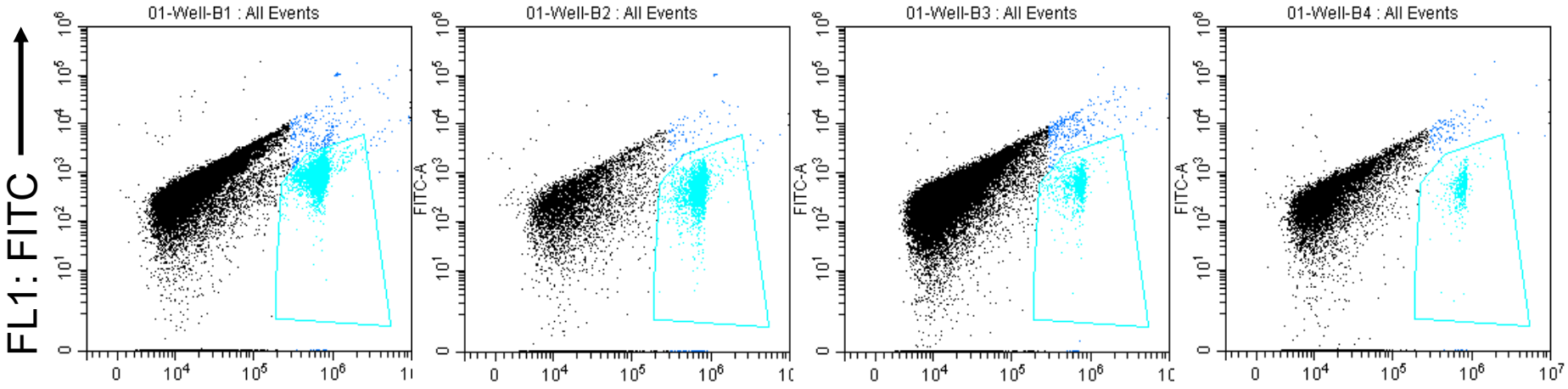
# Method Development Preliminary Results I

Quantification of total cells → DNA stain similar to previous methods

FL3: DNA staining →

FL1: FITC →




1'000'000/ml | 700'000/ml | 200'000/ml | 100'000/ml

→ DNA stained cells form a population that can be separated from the background in a 2D fluoresence dotplot.



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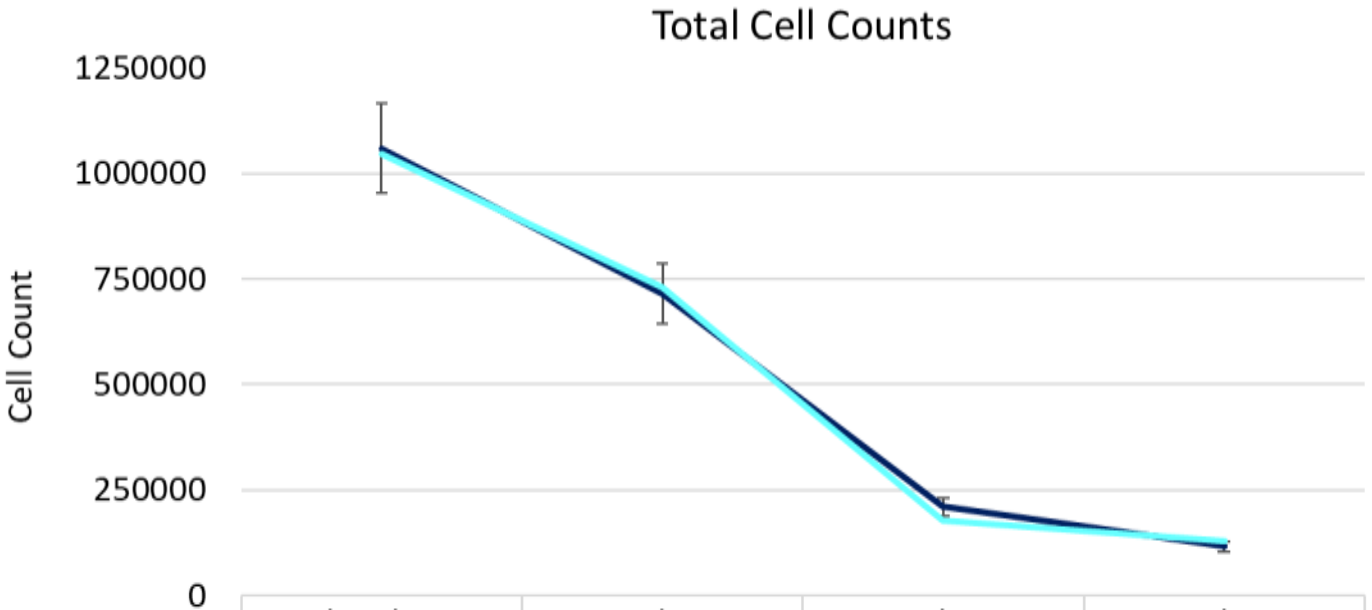
# Method Development Preliminary Results II

Quantification of **total cells → DNA stain**




| | 1'000'000 | 700'000 | 200'000 | 100'000 |
|---|---|---|---|---|
| target SCC | 1059000 | 716000 | 211070 | 116000 |
| DNA staining | 1046080 | 730820 | 177400 | 128200 |

→ Quantification of total cell counts is based on nuclear staining allowing the inclusion of every cell type present in milk

Error bars on target values 10%



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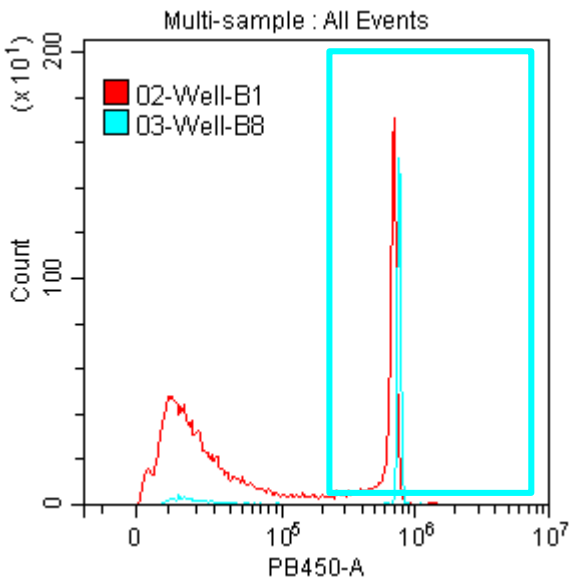
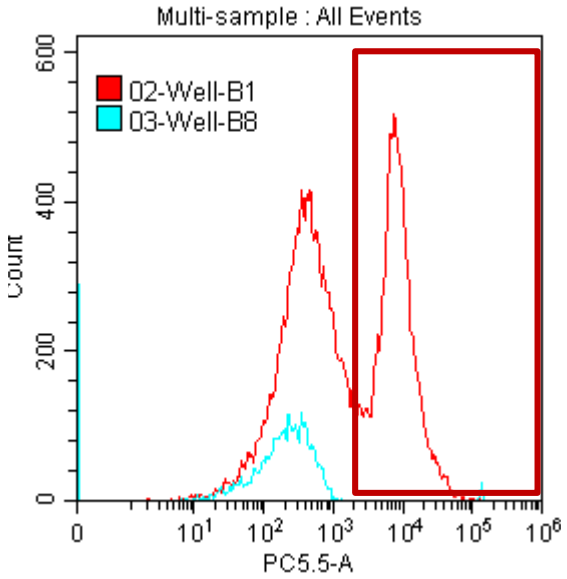
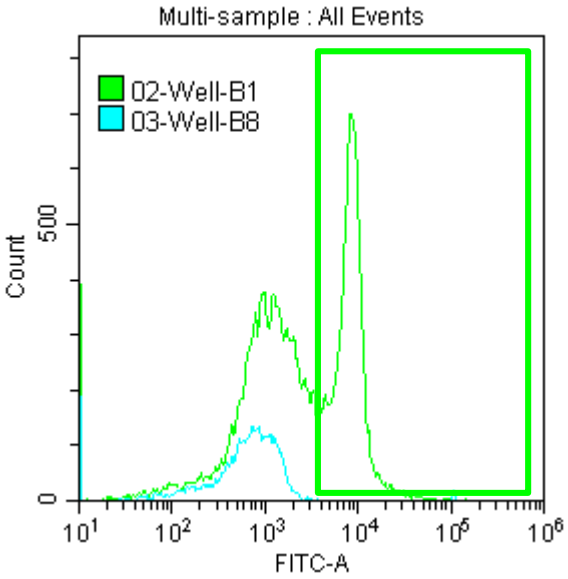
# Method Development Preliminary Results III

**Cell differentiation** with **specific antibodies** in the same run
→ Proof of principle with cells alone

Cell Counts




FL3:
DNA → total SCC

FL2:
Macrophages

FL1:
PMNs + Lymphocytes

Red: Cells in raw milk, double stained for DNA and an anti-macrophage antibody
Green: Cells in raw milk, double stained for DNA and anti-PMNs + Lymphocytes antibody
Blue: Cells only, single stain for DNA



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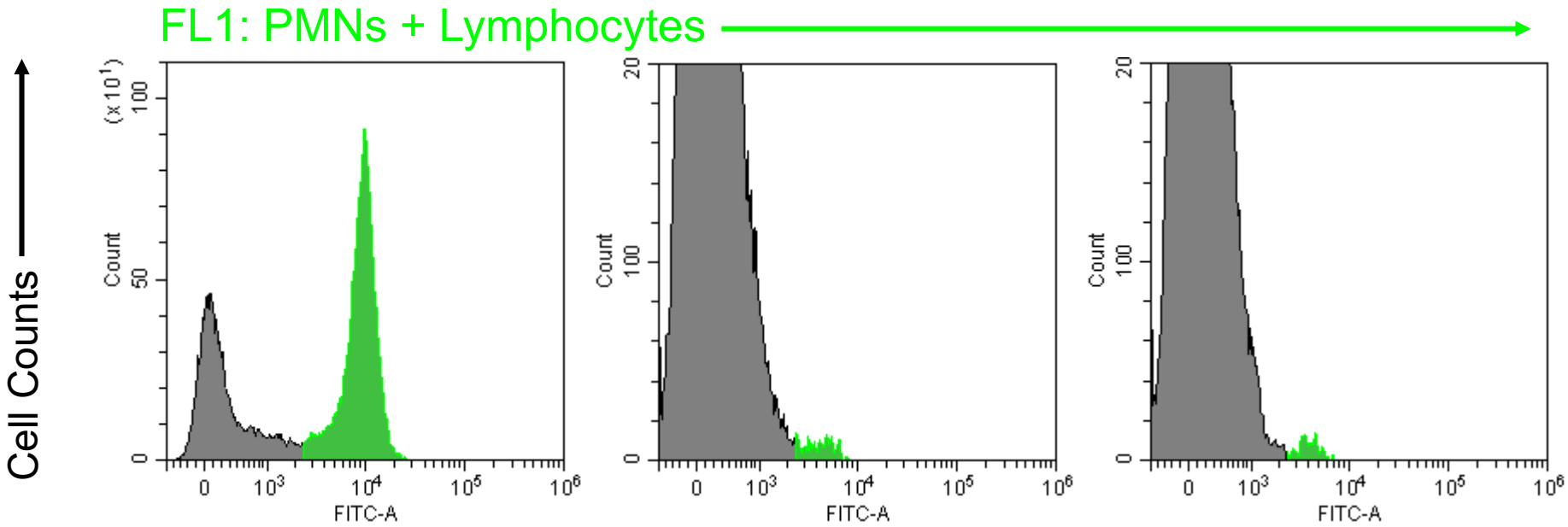
# Method Development Preliminary Results IV

Cell differentiation with specific antibodies in the same run




Stained isolated cells

Stained cells added to raw milk

Cells stained in raw milk

→ Isolated cell subpopulation (based on DNA) can be detected after antibody incubation in raw milk

# Further work

- Establish a specific double antibody pair for discrimination of PMN+Lymphocytes from macrophages in raw milk (CD14 that was previously used by Schwarz et al. is also present on milk fat globule membranes and is therefore not specific for the cells in raw milk)
- Reduction of background originating from milk fat globules to increase the sensitivity of the method by optimizing sample preparation prior to the analysis by flow cytometry
- Establish precision data for total and differential cell counts with the new method

# Summary

- Flow cytomtery allows the precise and reproducible quantification of total somatic cells in milk using DNA dyes
- Simultaneous DNA staining together with immunodetection using specific antibodies, total somatic cells and different subpopulations of interest can be quantified in one single flow cytometry experiment
- The presented method will be published, it is independent of a specific instrument, variable and adjustable for the specific needs for milk analysis in the future

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# Thank you for your attention

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