

M. Müller¹, S. Carstensen¹, C. Hennig², A. Mirenska², O. Holz^{1,3}, J.M. Hohlfeld^{1,3}

¹Fraunhofer ITEM - Hannover/DE, ²Zellkraftwerk - Hannover/DE,

³Member of the German Center for Lung Research (BREATH) - Hannover/DE



Introduction

Chipcytometry was developed for the analysis of cytometric and histological samples to understand structures, functions, and mechanisms on a cellular level. It combines the unsurpassed quantitative phenotyping ability of flow cytometry with the detailed imagery and functional insights of microscopy while maintaining cell and biomarker integrity over a storage period of several months. Induced sputum contains most valuable information on airway cell frequency and activation and is therefore a valuable target matrix for Chipcytometry. Sputum processing requires homogenization with the reducing agent DTT prior cellular analysis, which can affect surface marker expression (Loppow et al. ERJ 2000). We investigated whether standard treatment of sputum samples affects adhesion capability and stainability with standard surface marker antibodies. Furthermore, we assessed the effect of storage time on clinical samples in Chipcytometry slides.

Materials and Methods

Sputum samples and peripheral blood were collected from healthy subjects and COPD patients. **Flow cytometry:** PBMC and whole blood cells were incubated with 0.05 % DTT, 0.10 % DTT or PFA prior to antibody staining. PBS was used as control. The surface marker expression of CD45, CD3, CD4, CD8 and CD14 were investigated. **Chipcytometry:** The Zellscanner ONE® and specific Chipcytometry slides (Zellkraftwerk, Germany) with a cell-adhesive coating, which allows the unselective self-immobilization of cells by ionic bondings, were used (Fig 1). Cells on chips were stained and photobleached for up to 11 cycles using standard antibodies (PE-conjugated CD45, CD3, CD8, CD4, HLA-DR, CD14, CD16, CD123, CD25, CD69 and CD56).

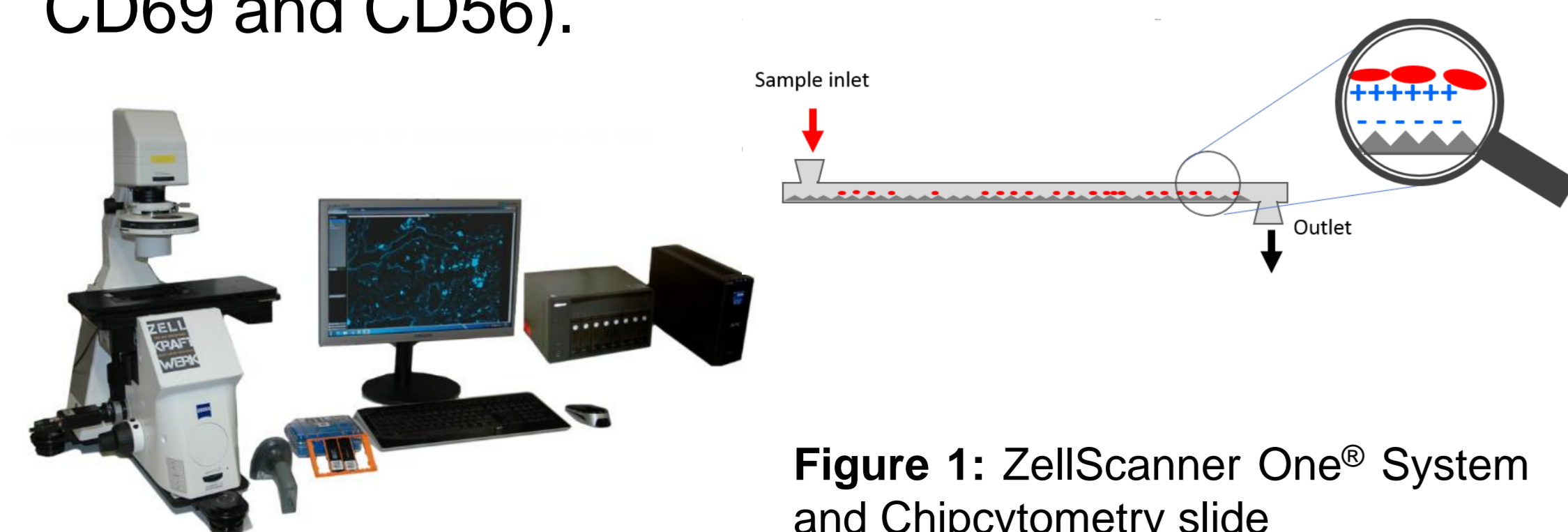


Figure 1: ZellScanner One® System and Chipcytometry slide

Loading and storage

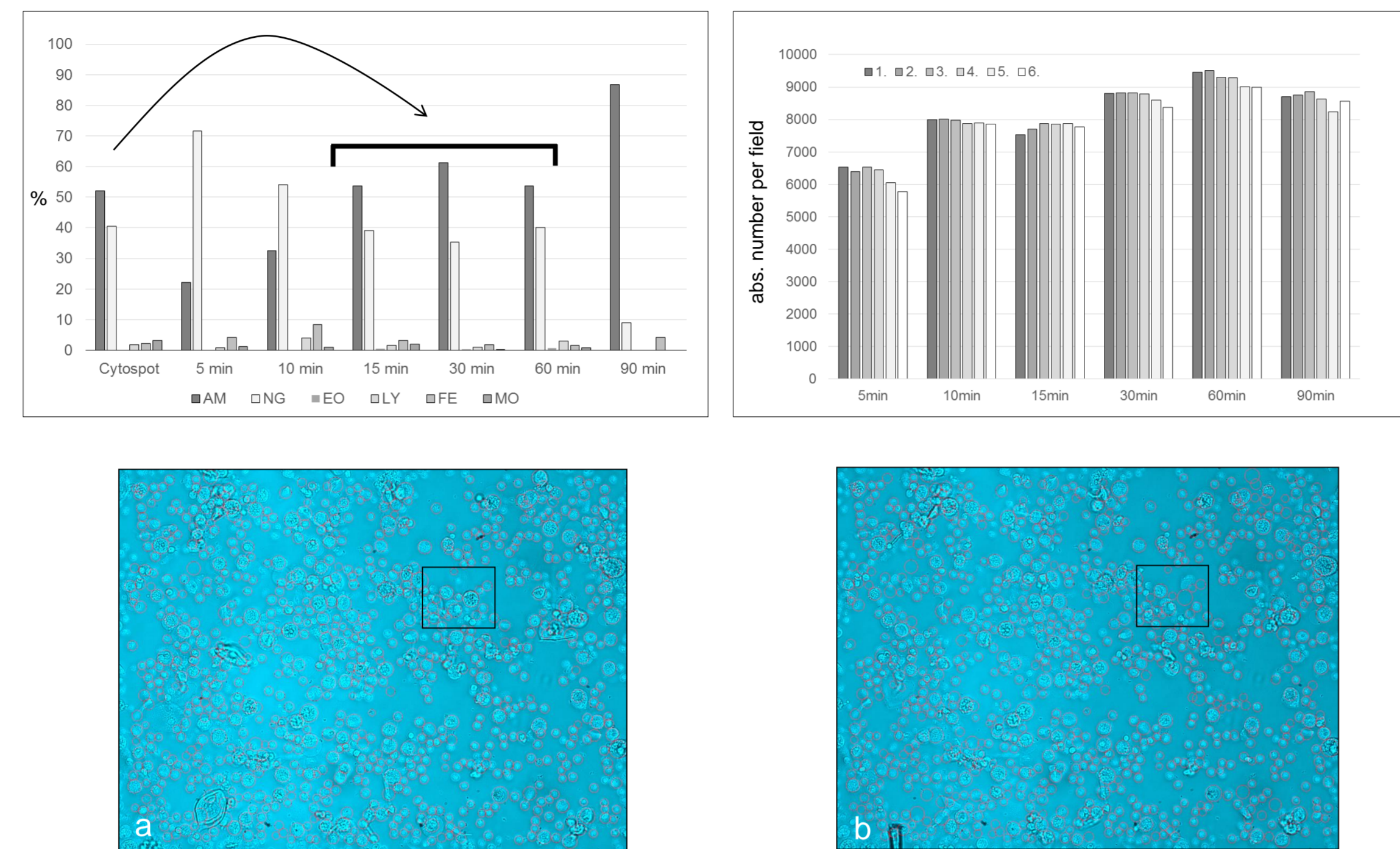


Figure 2: Cell density of sputum cells on Chipcytometry slide. Top left: The differential cell count (%) of the cytospot was comparable with the cell fraction which did not adhere to the slide and detached during the first wash after initial rest for 15-60 min, indicating that no selective cell loss occurs (AM: macrophages, NG: neutrophils, EO: eosinophils, LY: lymphocytes, FE: non-squamous epithelial cells, MO: monocytes). Top right: Cell loss over 6 staining cycles for chips with different loading incubation times after 2 days of storage. Similar results were obtained after 9 days of storage. Bottom: Transmitted light pictures of predefined positions were taken with every staining and bleaching cycle. Cell recognition algorithms determined cells after the first bleaching cycle. The segmentation is shown as red circles. (a) Cell loading density after the first staining. The algorithm detects most of the cells as single events. (b) Cell density after ten staining and bleaching cycles. Most of the cells remain on their position. Individual cells detached as can be seen for example in the area framed by the black square.

Effect of DTT

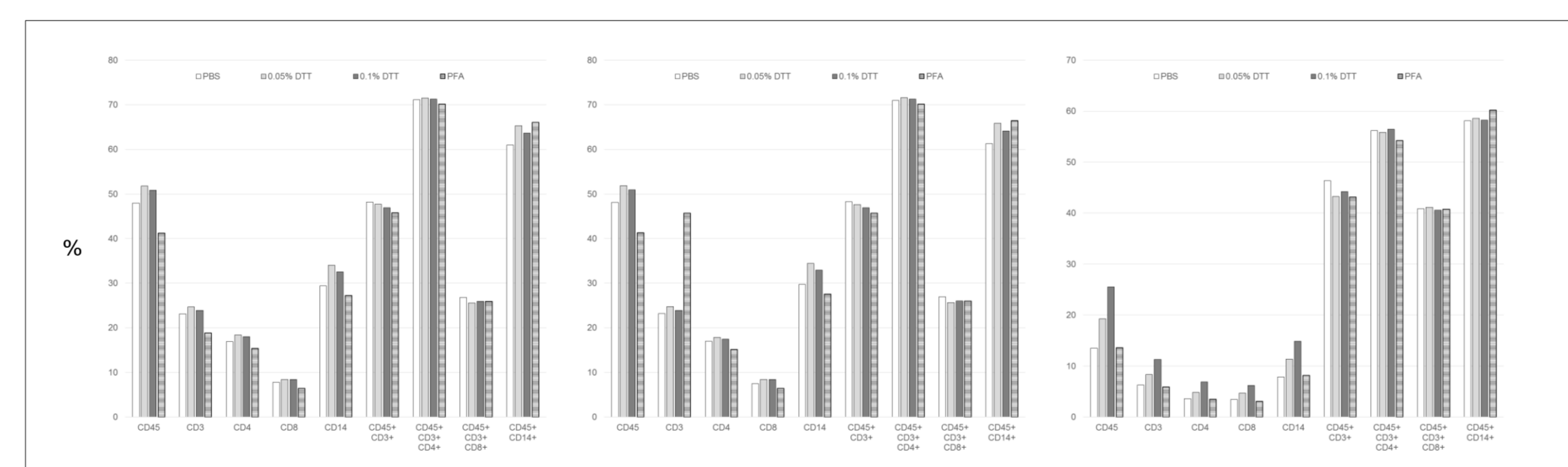


Figure 3: Effect of DTT on PBMCs isolated from peripheral blood of 3 subjects. Cells were treated like induced sputum samples and pre-incubated with the indicated solutions to test the effect of DTT and PFA (needed for fixation of cells on Chipcytometry slides) on flow cytometric measurements of selected cell surface markers.

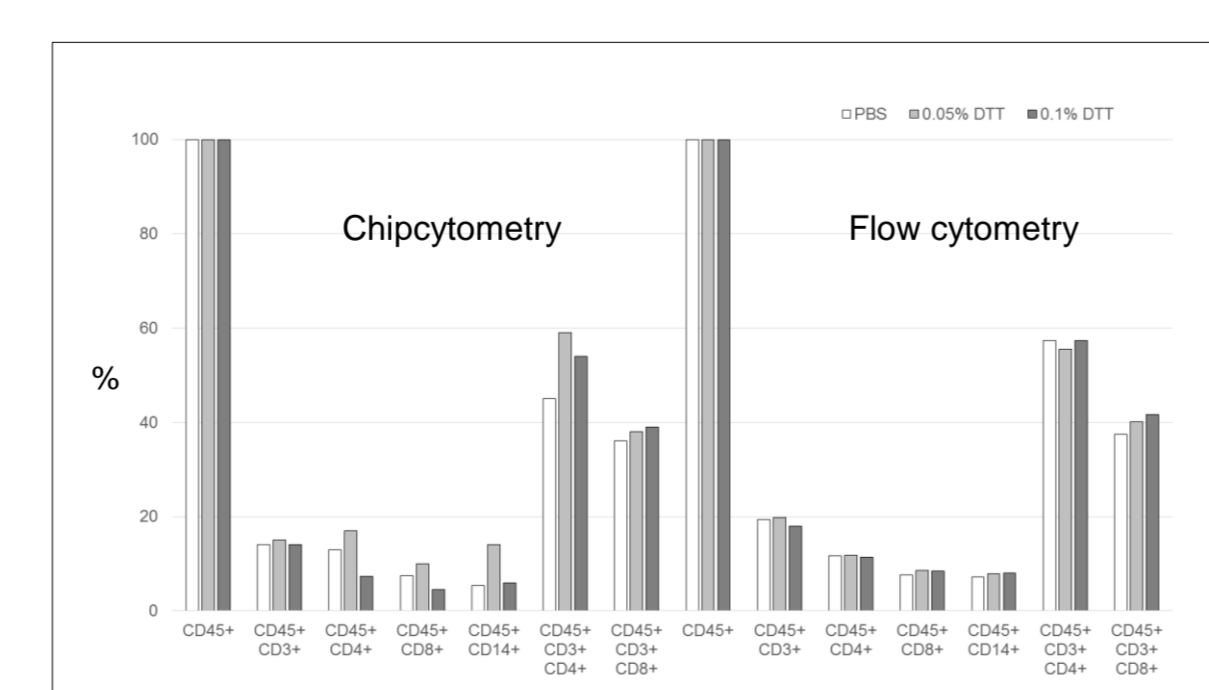


Figure 4: Effect of DTT on antibody staining of whole blood cells. Data of 1 subject was analyzed both with Chipcytometry and with flow cytometry.

Staining of sputum samples with Chipcytometry

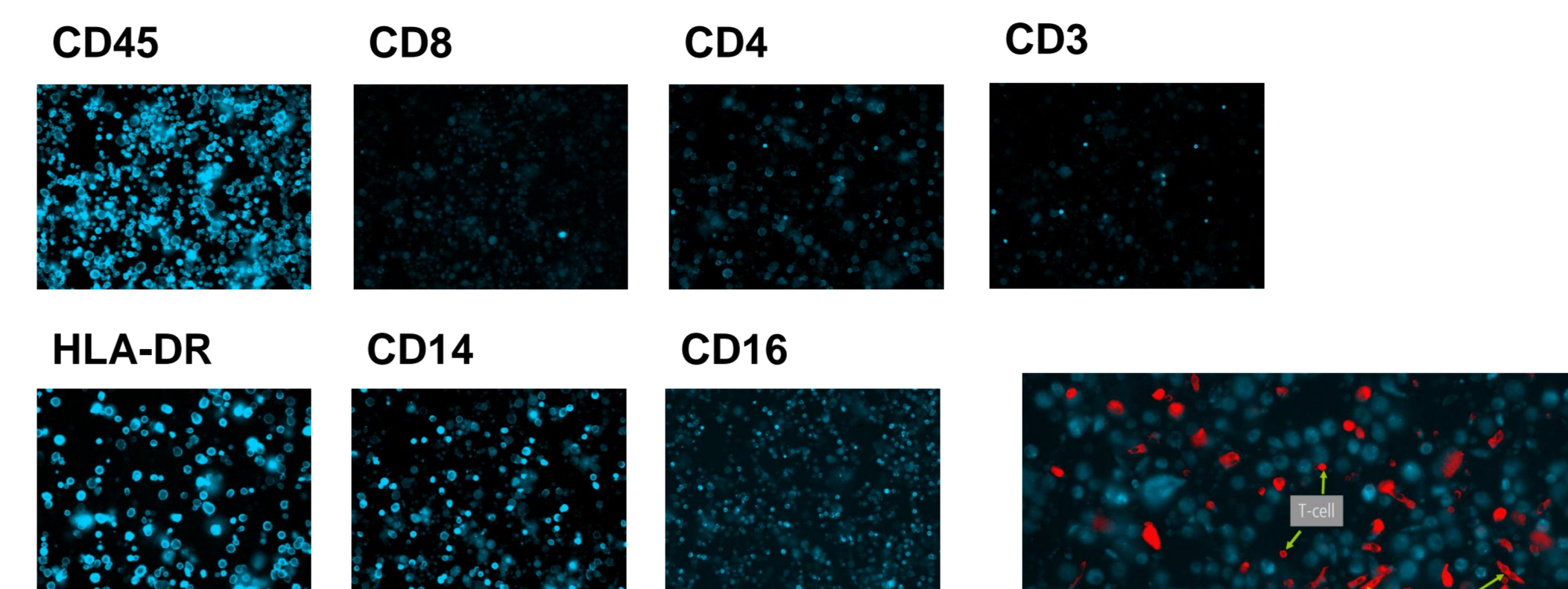


Figure 5: Examples for cell surface staining of sputum cells by Chipcytometry. The CD3 clone used showed unspecific staining to non-squamous epithelial cells in a subject with higher percentage of these cells.

Autofluorescence

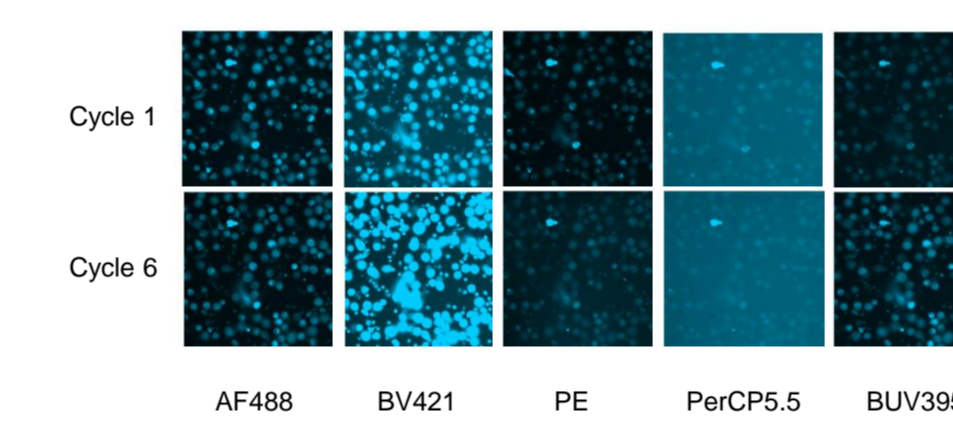


Figure 6: Autofluorescence of sputum cells of a healthy subject at different wavelengths (indicated by the typical color used).

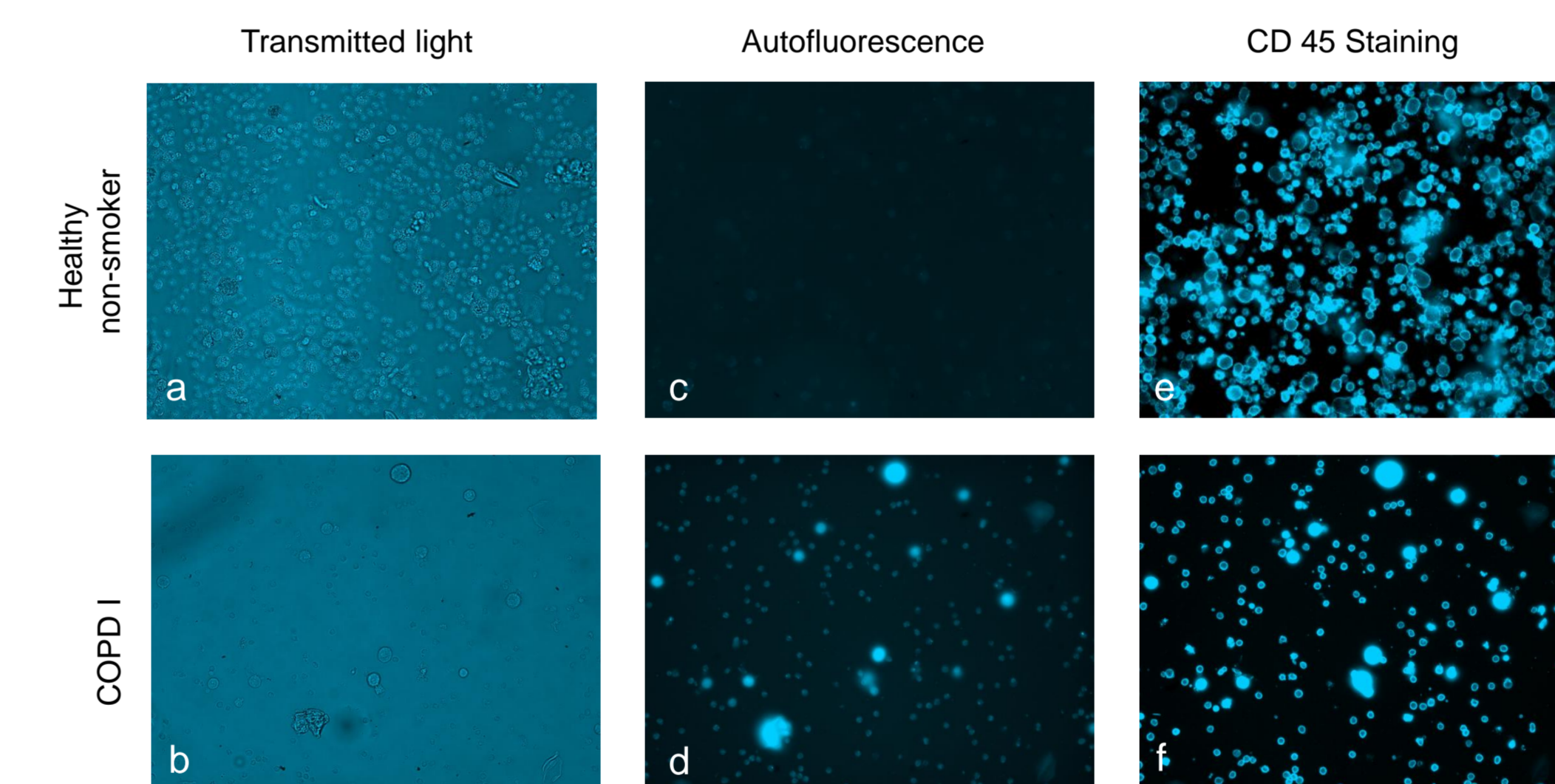


Figure 7: Transmitted light (a, b), autofluorescence and Phycoerythrin (PE)-fluorescent pictures of healthy and COPD I donor sputum samples. (c, d) Autofluorescence of healthy macrophages and granulocytes is lower compared to the COPD patient. (e, f) Staining with anti-CD45 PE antibody.

Selected findings

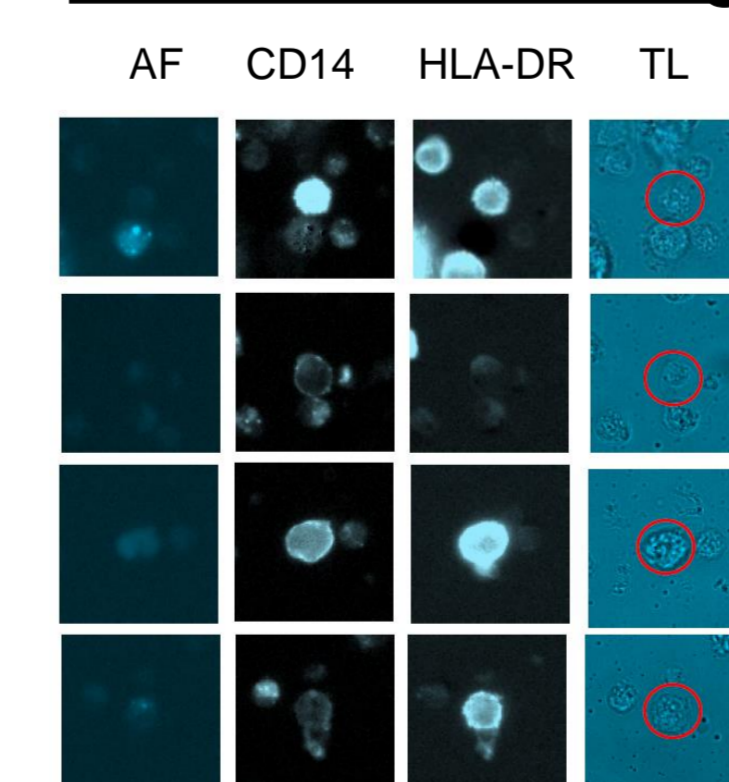


Figure 8: CD14 / HLA-DR staining: Cells of variable morphology are either CD14 single or HLA-DR single or CD14/HLA-DR double positive. AF: autofluorescence, TL: transmitted light.

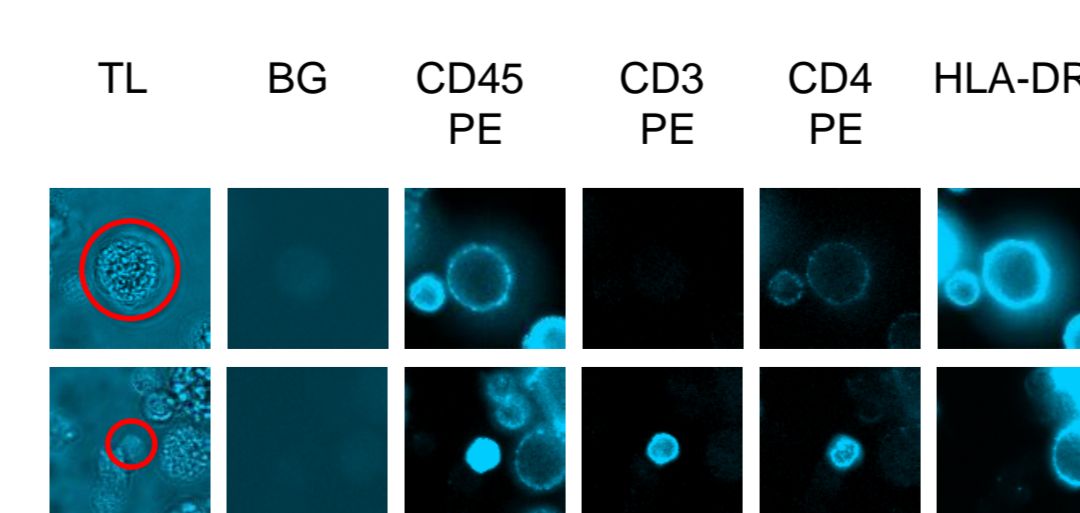


Figure 9: A cell with macrophage morphology is CD45 and HLA-DR positive, a cell with lymphocyte morphology and size shows CD3 and CD4 staining. BG: background

Results

Homogenized sputum samples could be loaded in acceptable numbers on Chipcytometry slides. The cell load protocol had to be optimized to sputum cells and requires a minimum attachment time of 15 min. (Fig. 2). Chipcytometry analysis up to 9 days of storage at 4°C, showed that >98 % of the sputum cells remained on the chip following 6 staining and bleaching cycles using an adapted sputum staining/washing protocol (Fig.2). Using antibody panels this allows staining of max. 25 different antibodies. First qualitative data suggest that storage times of sputum chips up to 7 months are possible. For the antibodies tested so far, the treatment with DTT did not affect the epitopes on PBMCs when tested by flow cytometry (Fig. 3). Comparable results were obtained with whole blood samples using flow cytometry and Chipcytometry (Fig. 4). Unspecific binding of CD3 was observed on bronchial epithelial cells (Fig. 5). Autofluorescence on cells was observed in different degrees in the available light channels (Fig. 6) and often a higher level in COPD patients (Fig. 7). Slides loaded with cells of sputum with poor quality (large proportions of squamous cells) or a higher proportion of degraded cells were more difficult to evaluate. (Fig. 8+9) show multiple stainings for different cell types.

Conclusion

These first Proof of Principle experiments demonstrated that the cellular analysis of sputum by Chipcytometry is possible. We show first results for the description of cellular populations. High quality sputum samples and careful preparation with low squamous cell contamination will be a prerequisite for this analysis. Short term storage did not affect the analysis of the samples which allows multicenter trials with centralized analysis of sputum by Chipcytometry. Further adaptation and fit-for-purpose validation of the method for induced sputum samples is required.

Contact

Fraunhofer ITEM. Hannover. Germany
Meike.Mueller@item.fraunhofer.de
www.item.fraunhofer.de