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Research paper

Comparison of immunophenotyping by slide-based cytometry and by flow cytometry☆

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Abstract

Background: Flow cytometry (FCM) is the gold standard for immunophenotyping of peripheral blood leukocytes (PBLs). Slide-based cytometry (SBC) systems, for example the laser scanning cytometer (LSC®, CompuCyte), can give additional information (repeated staining and scanning, morphology). In order to adequately judge the clinical usefulness of LSC for immunophenotyping it is obligatory to compare it with FCM.
Aim: The aim of this study was to systematically compare immunophenotyping by both FCM and LSC methods and to test the correlation of the results.
Methods: PBLs were stained with directly labeled monoclonal antibodies with the whole blood staining method. Aliquots of the same paraformaldehyde fixed specimens were analyzed in parallel by a FACScan (BD-Biosciences) using standard protocols and by LSC with different triggers (forward scatter, CD45 FITC, or 7-AAD). For 7-AAD measurements by LSC, slides were additionally fixed with acetone before 7-AAD staining.
Results: Calculating the percentage distribution of PBLs obtained by LSC and by FCM showed very good correlation with regression coefficients close to 1.0 for the major populations and the lymphocyte sub-populations (neutrophils, monocytes, and lymphocytes; T-helper-, T-cytotoxic-, B-, NK-cells). The best trigger for LSC was 7-AAD.
Conclusion: LSC can be recommended for immunophenotyping of PBLs especially in cases where only limited sample volumes are available or where additional analysis of the cells' morphology is important. The detection of rare leukocytes or weak antigens is limited; in these cases appropriate amplification steps for immunofluorescence should be engaged.

Keywords: Laser scanning cytometry (LSC); Flow cytometry; Immunophenotyping; Triggering; Comparative study; Resolution parameter

Abbreviations: 7-AAD, 7-aminoactinomycin-D; FCM, flow cytometry; FSC, forward scatter; LSC, laser scanning cytometry; PBLs, peripheral blood leukocytes; PMT, photomultiplier; SBC, slide-based cytometry.

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1. Introduction

Flow cytometry (FCM) is the gold standard for immunophenotyping of PBLs. This is due to the high throughput, the multi-parametric analysis, the possibility of detecting rare events and weak signals, and the standardized preparation and analysis protocols worldwide (Borowitz et al., 1998). However, there are specific problems that can only be incompletely addressed by FCM, if at all. A major problem in clinical samples from pathologically altered material is the fact that, in general, analysis of the cells' morphology by FCM is limited to forward and orthogonal side scatter. After analysis by FCM, morphology can usually be studied only by cell sorting which is time-consuming but still does not allow correlations with the fluorescence data on a single-cell basis. Morphology, however, is a very important feature especially in pathological conditions and therefore it should be available for thorough analysis. This gave rise to the demand for technologies that combine both multiparametric analysis and documentation of cell morphology (Kachel et al., 1997). This was recently made commercially available by an instrument applying spectral decomposition and double-illumination by brightfield and 488 nm laser (George et al., 2004). It allows documentation of the morphology of cells in greater detail than was previously possible with flow instruments. Other novel instrumentation use microfluidic chip-technology (Dürr et al., 2003; Hughes, 2002; Palková et al., 2004). In particular these devices miniaturize the amount of sample material to be analyzed.

However, a problem unsolved by all instruments analyzing cells in solution is the fact that cells cannot be analyzed a second time on a cell-to-cell basis. This is important, e.g., in order to maximize the information content extracted from a minimal sample as it could be achieved, e.g., by applying a new fluorescence staining for different cell characteristics. In clinical practice, this might be crucial in the case of critically ill neonates or in fine needle aspirate biopsies.

Slide-based cytometry (SBC) is an alternative approach to fill the gap between high throughput multiparametric cytometry on the one hand and detailed morphological analysis and documentation on the other hand (Tárnok and Gerstner, 2002). The first commercially available SBC instrument was the LSC (CompuCyte Corp., Cambridge MA) (Kamentsky and Kamentsky, 1991). The instrumentation and software of the LSC is explained elsewhere (Kamentsky and Kamentsky, 1991; Kamentsky et al., 1997; Martin-Reay et al., 1994; Tárnok and Gerstner, 2002) and is described only briefly. The instrument is built around a routine epifluorescence microscope equipped with a motorized stage and up to three single lasers (405 nm violet diode laser, 488 nm argon-laser, 630 nm helium–neon-laser). The cells are immobilized on a glass slide and scanned. The emitted fluorescence is guided to four optical filter sets coupled to four photomultipliers (PMTs). In addition, the light scattered by the cells is detected by a photodiode underneath the slide producing a signal called forward scatter (FSC). A digital image is created for each PMT on a pixel-to-pixel basis. These images are analyzed applying a trigger signal defined by the operator that could be any fluorescence or the FSC as well as combinations of both. A number of data sets are then acquired per object such as integral fluorescence and Max Pixel, equivalent to fluorescence integral and height in FCM, respectively. The LSC allows analysis of up to 5000 cells per minute. In the meantime, some more slide-based cytometric instruments have become commercially available (Bocsi et al., 2004; Hoetzenecker et al., 2005).

The most important feature of LSC is that the exact position of every object is recorded together with the fluorescence data and therefore each object can be directly visualized at any time after completing the analysis. Amongst other things this makes it possible to verify whether objects are single cells, doublets, debris, or artifacts, and to document the cells' morphology. To this end the slide can be removed from the stage, stained by conventional cytological methods (Giemsa, Hematoxylin and Eosin), and placed on the stage again (Gerstner et al., 2000, 2002a). In addition, this "no loss" design is a prerequisite for the analysis of hypocellular specimens.

One of the first clinical applications of LSC was immunophenotyping of PBLs (Clatch and Walloch, 1997; Clatch and Foreman, 1998; Gerstner et al., 2000). We have shown that the system can be used to detect up to six different fluorochromes simultaneously using near-infrared dyes (Gerstner et al., 2002b), and recently its capacity was further increased to eight dyes (Mittag et al., 2005). In order to adequately judge the clinical usefulness of immunophenotyping by LSC it is obligatory to compare it with the long established FCM assays. We therefor performed this study to systematically compare immunophenotyping by the two methods, FCM and LSC, and to test their correlation. We used different parameters as trigger signals to detect leukocytes in the samples: nuclear DNA stains (Gerstner et al., 2000), light scatter (Gerstner et al., 2002b) or panleukocyte surface antigen staining with anti-CD45 antibody (Clatch and Foreman, 1998). We also