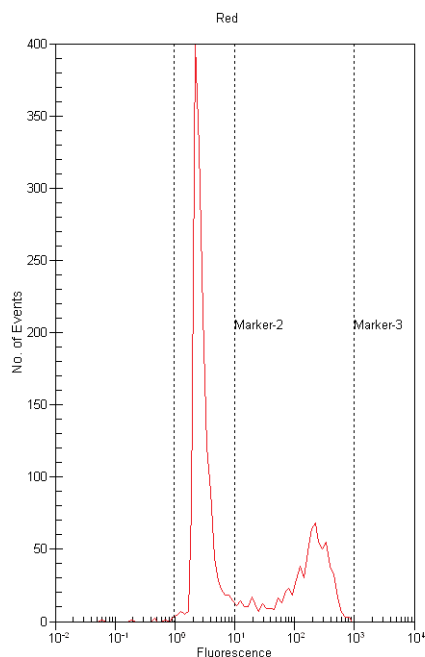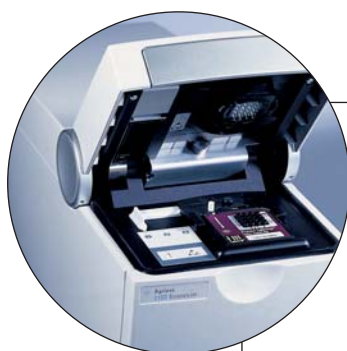# Identification of red and white blood cells from whole blood samples using the Agilent 2100 bioanalyzer

Application Note

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Abstract

This Application Note describes an antibody based method to identify and count blood cells from whole blood samples on the Agilent 2100 bioanalyzer without a washing step.

The ability of the Agilent 2100 bioanalyzer to identify peripheral whole blood cells is demonstrated and compared to a flow-cytometry-based reference instrument.

Agilent Equipment:

2100 bioanalyzer
Cell fluorescence assay kit

Application Area:

Blood cell analysis

Agilent Technologies

Introduction

This Application Note describes a method to identify blood cells from a whole blood sample using antibody staining and cell fluorescence assays on the Agilent 2100 bioanalyzer without a washing step. A broad variety of cell lines have been tested with the Agilent 2100 bioanalyzer so far. We want to extend these tests to whole blood specimens. Suspended in the aqueous plasma there are seven types of blood cells and fragments:

- Red blood cells (RBCs or erythrocytes)
- Five types of white blood cells (WBCs or leucocytes)
- Platelets (or thrombocytes)
- The cells (mainly RBCs) constitute 45 % of the blood volume equivalent to 4 million cells per µL for normal blood. WBCs are much less numerous, the ratio between WBCs and RBCs is around 1:700.

The experiments were performed to identify several blood cell populations by defining staining protocols for the separation and identification of RBCs, whole WBCs and leucocyte subpopulations, such as monocytes or granulocytes. Nucleic acid dye and antibody staining was used in accordance with the dual-fluorescence detection system of the Agilent 2100 bioanalyzer (λ_{ex} 470 nm / λ_{em} 525 nm and λ_{ex} 635 nm / λ_{em} 680 nm).

Experimental

The blood samples used were human venous blood collected directly in vacutainer plastic tubes containing EDTA-K3 as anticoagulant.

Staining of red blood cells

The monoclonal antibody anti-human CD235a conjugated with the fluorochrome Alexa 647, a red excited fluorescent marker (Abd-Serotec), was chosen for RBC staining. Each sample was diluted to adjust the cell density with the Agilent cell buffer, a component of the cell kit. For prestaining the RBCs, 50 µL of diluted (1:600) whole blood were transferred into a tube containing 15 µL of monoclonal antibodies. After 30 minutes of incubation at room temperature in the dark, the sample was diluted (1:50) and then dispensed into the chip wells.

RBCs staining protocol

1. Dilute 5 µL of blood sample with 3 mL of Agilent cell buffer.
2. Add 15 µL of the antibody (anti-CD235a) to 50 µL of diluted blood.
3. Incubate for 30 minutes at room temperature in the dark.
4. Dilute 10 µL of the stained blood sample with 500 µL of Agilent cell buffer and use 10 µL as sample.
5. Prepare and run the chip in the Agilent 2100 bioanalyzer according to the reagent kit guide.

Staining of white blood cells

To be able to study WBCs from whole blood samples a red cell lysing step is needed in the prestaining protocol. The lysis reagent, formulated for an optimal erythrolytic effect, contains 3 % (w:v) of saponine in a 1 % paraformaldehyde solution. This solution lyses the RBCs with a minimal effect on WBCs. To stain the WBCs a nuclear acid dye such as SYTO 16 (blue excited fluorescent marker obtained from Invitrogen/Molecular Probes) was used.

WBCs staining protocol

1. Add 2 µL of SYTO 16 (1 mM) to 20 µL of whole blood.
2. Wait 5 minutes and add 200 µL of lysing solution. Incubate 30 minutes at room temperature in the dark.
3. Dilute the preparation (1:2) with the Agilent cell buffer.
4. Load and run the chip in the 2100 bioanalyzer according to the reagent kit guide.

Staining of WBC subpopulations

To study WBC subpopulations without using a ficoll-like preparation, the prestaining cell protocol still includes the lysing step. Two types of fluorescent markers, a blue dye and red fluorescent monoclonal antibodies were used for these stainings. The monoclonal antibodies anti-human anti-CD14 and anti-CD16, both conjugated with the fluorochrome PE-Cy5, were purchased from Beckman Coulter.

WBC subpopulations staining protocol

1. Add 2 µL of anti-human CD PE-Cy5 (1 mg/mL) to 20 µL of whole blood and incubate for 25 minutes at room temperature in the dark.
2. Add 2 µL of SYTO 16 (1 mM) and incubate for 5 minutes at room temperature in the dark.
3. Add 200 µL of lysing solution and incubate 30 minutes at room temperature in the dark.
4. Dilute the preparation (1:2) with the Agilent cell buffer.
5. Load and run the chip in the Agilent 2100 bioanalyzer according to the reagent kit guide.

Results and discussion

The detection of blood cells was performed in combination with the cell chip kit and the Agilent 2100 bioanalyzer expert software for flow cytometry. Data acquisition was performed with no requirement for specific parameters. Conventional flow cytometry of immunophenotyping assays involves the identification of cells by combining their light-scattering properties with surface antigen binding to fluorochrome-conjugated monoclonal antibodies. In this study, we show that the Agilent 2100 bioanalyzer is able to analyze a whole blood sample and to identify different blood cell populations such as RBCs, total WBCs and also WBC subpopulations, without the need for scattering data.

Staining of red blood Cells

The red fluorescence intensity of the antibody staining of RBCs was low but nevertheless sufficient enough to mark the whole red cell population, (figure 1A). Due to their small size 2500-3000 events were analyzed. To achieve actual counts/mL of RBC's it is possible to use fluorescent beads (reference FXC R98.5 from Merck-Estapor) for an evaluation of the cell number using a 80:20 ratio (cells:beads). The cell counting data from the Agilent 2100 bioanalyzer is within a XY% interval as compared to an established cell counting system (figure 1B).
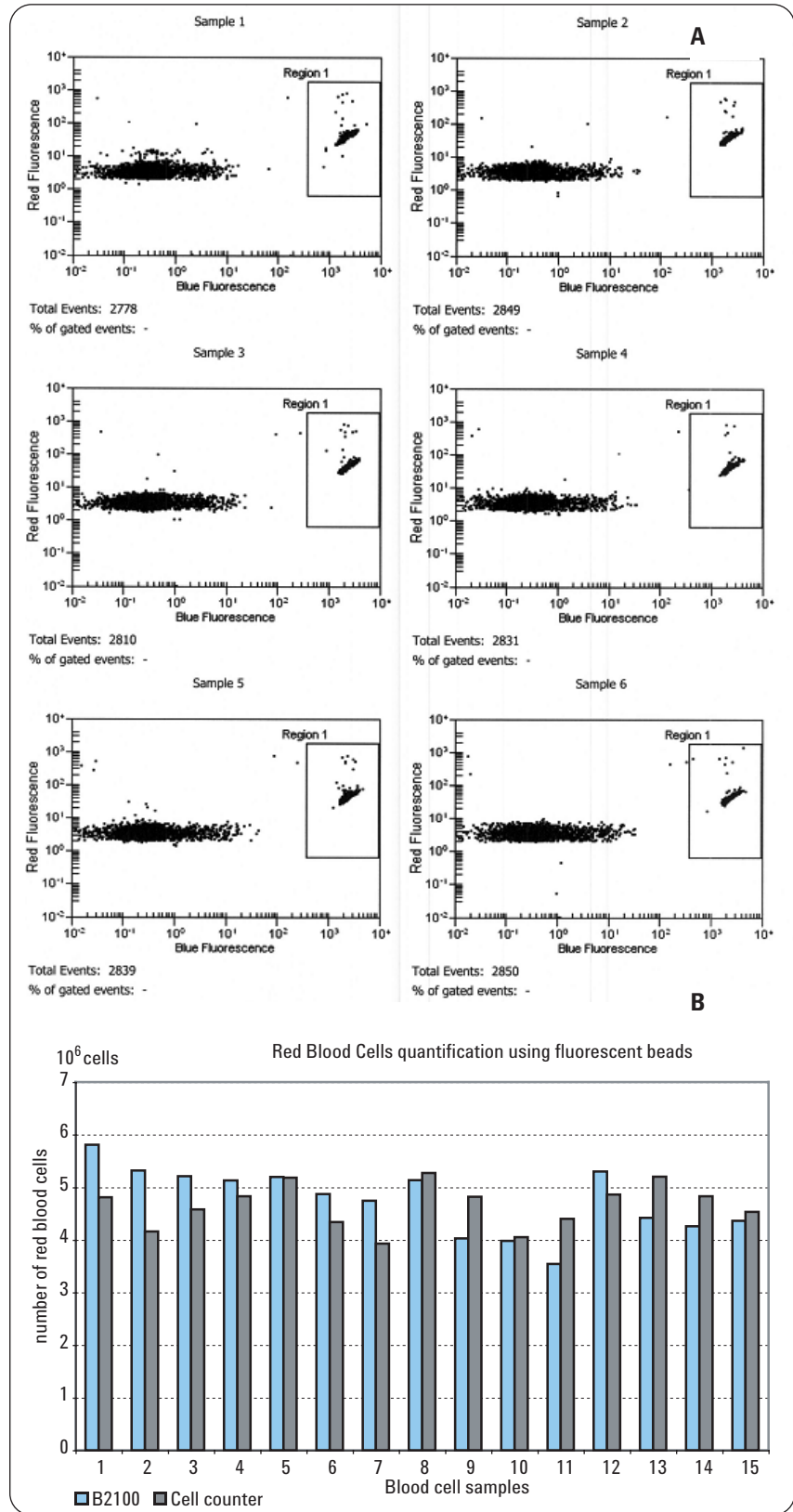

Figure 1
Red blood cells – A) shows on-chip antibody staining results as dot plots for 6 samples. The RBCs are stained with an anti-CD235a monoclonal antibody conjugated with the fluorochrome. B) shows the quantitative determination of RBC counts using the Agilent 2100 Bioanalyzer versus automatic blood cell counter (H-ABX). The double-fluorescent bead population gated in the region 1 can be used as a reference for cell counting.

Staining of white blood cells

The monofluorescent staining using nuclear acid dye SYTO 16 was performed to identify the total WBCs (figure 2).
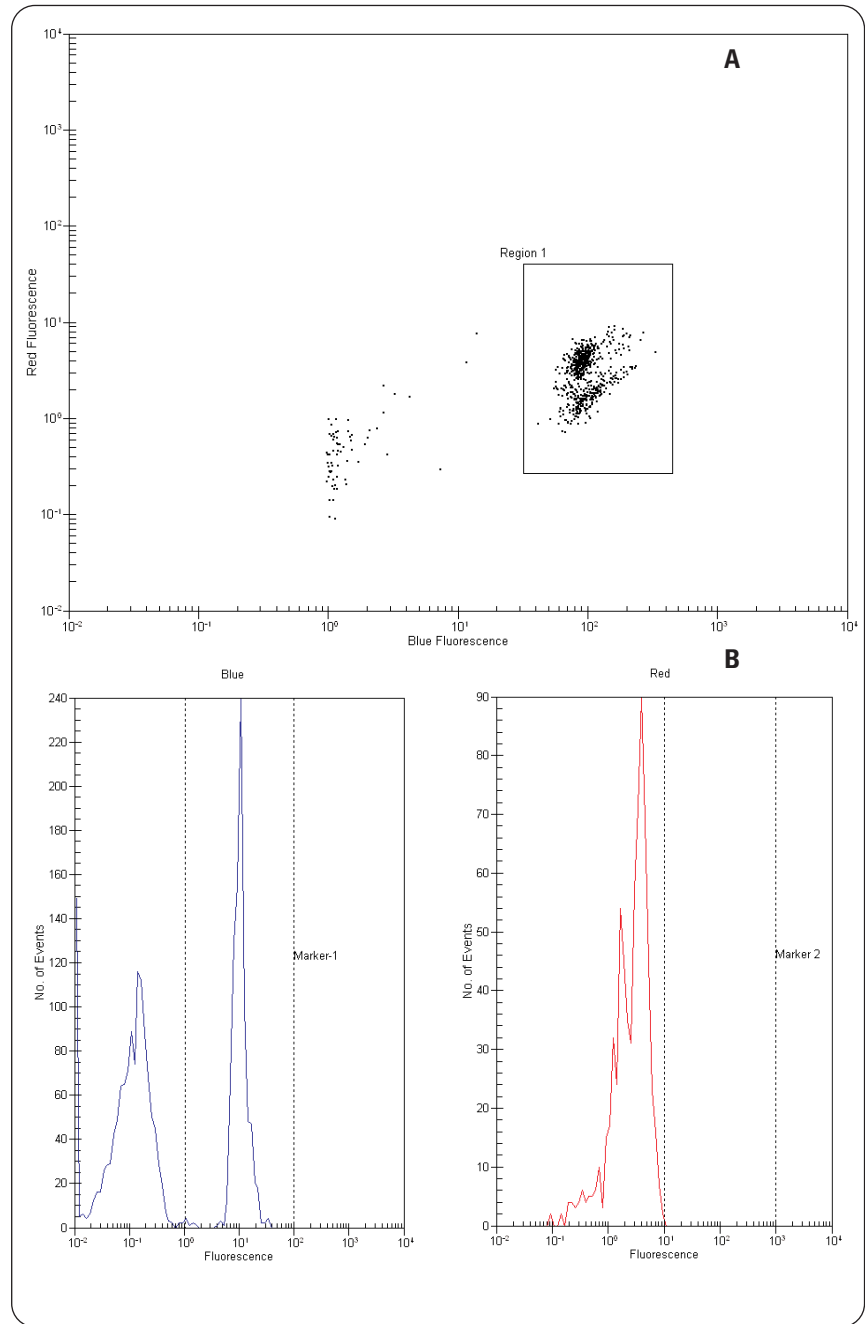

Figure 2
White blood cells – The dot-plot (A) and histogram (B) illustrate the SYTO 16 intracellular staining of total leucocytes.
To eliminate some residual red cell debris, WBCs are gated on the dot-plot in the region 1 and are seen in the Marker 1 on the blue fluorescence histogram.

For WBC subpopulations a dual fluorescent staining with SYTO 16 and PE-Cy5 monoclonal antibodies was done. Anti-CD16-PE-Cy5 was used for the granulocyte identification (figure 3).
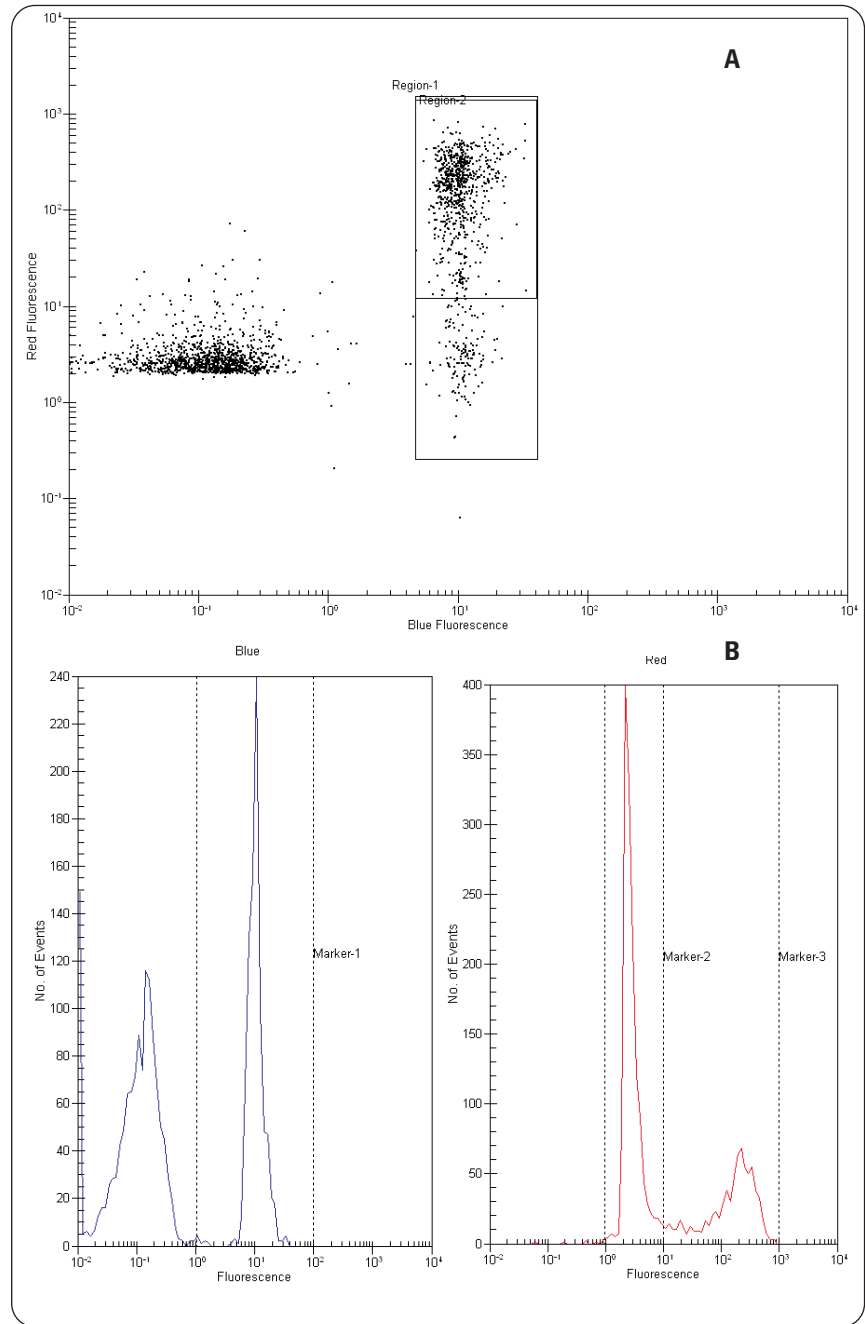

Figure 3
Granulocytes – The CD-16 antigen [FC receptor for immunoglobulin G (Fc_RIII)] is mainly expressed on granulocytes first at the metamyelocytic stage and is maintained throughout granulocyte maturation. The SYTO 16 stained all the WBCs as shown in Region 1 on the dot plot (A). The red fluorescence of the antibody staining (anti-CD16 PE-Cy5) identifies the granulocyte population (B: Marker 3 on histogram).

Anti-CD14-PE-Cy5 was used for the monocyte identification (figure 4). When comparing the WBC cell counting data obtained with the Agilent 2100 bioanalyzer to an established cell counting system the Agilent 2100 bioanalyzer is within a XY % interval (figure 4B).
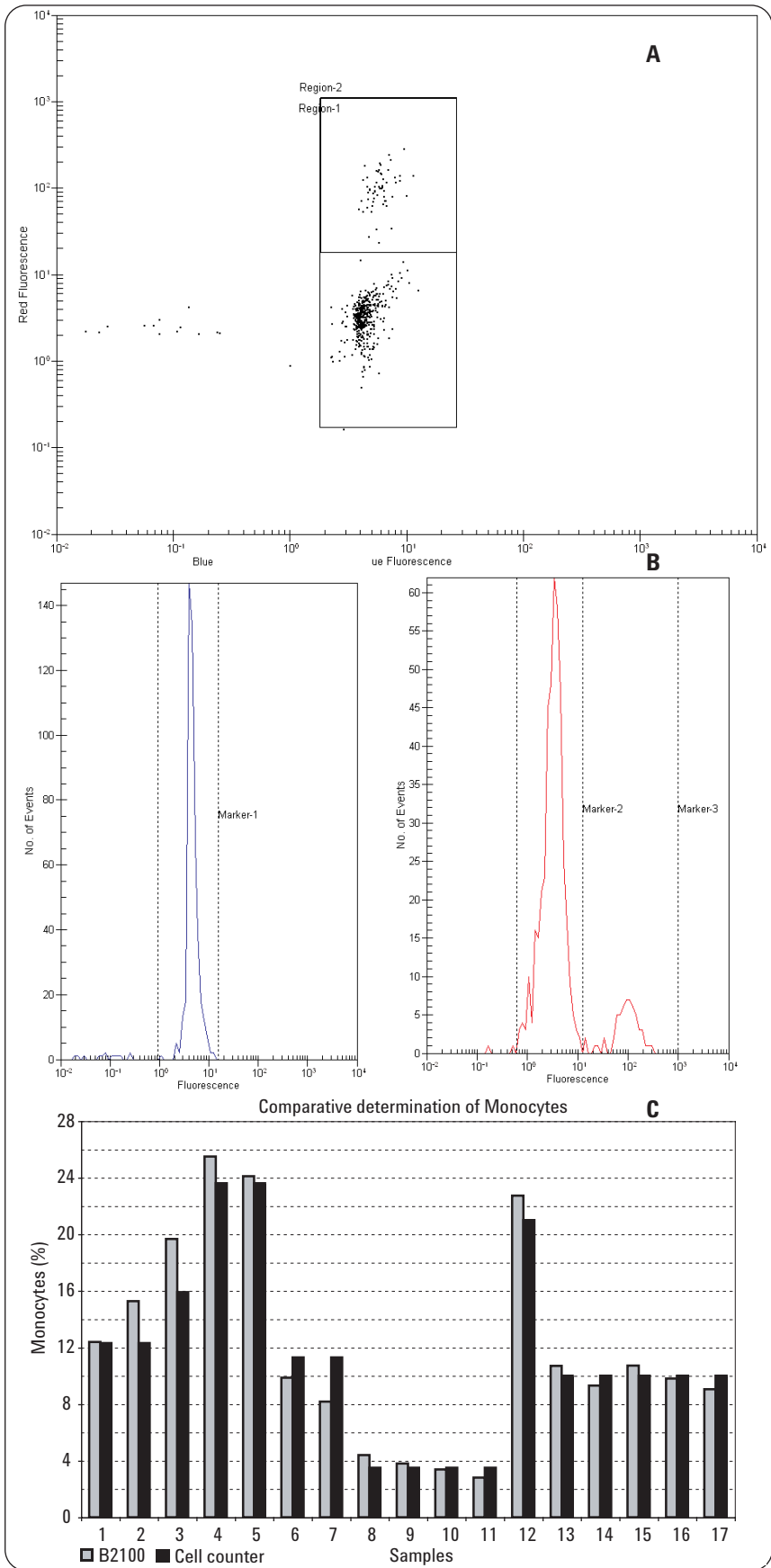

Figure 4
Monocytes – The antigen CD14 is a glycolipid-anchored membrane glycoprotein expressed on cells of the myelomonocyte lineage including monocytes and macrophages. They function as receptors for the complex of lipopolysaccha-ride (LPS) and LPS-binding protein. The SYTO 16 stained all the WBCs as shown in Region 1 on the dot plot (A). The red fluorescence of the antibody staining (anti-CD14-PE-Cy5) identi-fies the monocyte population – Region 2 on the dot-plot (A) or Marker 3 on the red fluores-cence histogram (C). In (B) a comparative determination of monocytes between the Agilent 2100 bioanalyzer and an automatic blood cell counter is shown.

Conclusion

The ability of the Agilent 2100 bio-analyzer to identify peripheral whole blood cells using nuclear acid dyes and/or monoclonal anti-bodies has been tested successful-ly on-chip. In comparison with an automatic flow-cytometry-based instrument (cell counter), the micro-volumes of samples and reagents, the low number of cells required for the analysis (only 30000 cells per sample) as well as the easy use of the Agilent 2100 Bioanalyzer are the specific advantages of this microfluidic chip-based technology. These advantages can be used for the analysis of small sample volumes or in particular for the analysis of some blood samples low in cell numbers.

References

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