Validation of the appearance position of leukocytes in Sysmex's haematology automated analyser

The Cell Analysis Center – Scientific Bulletin Part 6

In Scientific Bulletin Part 3, about the reticulocyte measurement functionality [1] of Sysmex's XE Family of automated haematology analysers, it was explained that the analyser accurately identifies and counts the reticulocytes by three methods, (a) labeling[1] CD71[2], which is a marker[3] of an immature erythrocyte with FITC[4], (b) staining with Sysmex's specific reagent for reticulocyte measurement RET Search (II) and (c) staining with new methylene blue [2]. In Part 4 and Part 5, regarding leukocyte differentiation measurement functionality [5], it was explained how the reagents Stromatolyser-4DL (surfactant) and Stromatolyser-4DS (fluorescent dye) stain and identify the leukocytes inside the analyser. In this bulletin, we could verify that the appearance position of various types of leukocyte cells separated using a CD antibody which specifically[5] recognizes each leukocyte using the same method as described in bulletin Part 3, was the same as compared to the appearance position on a leukocyte differentiation scattergram[6] of Sysmex's haematology analysers.

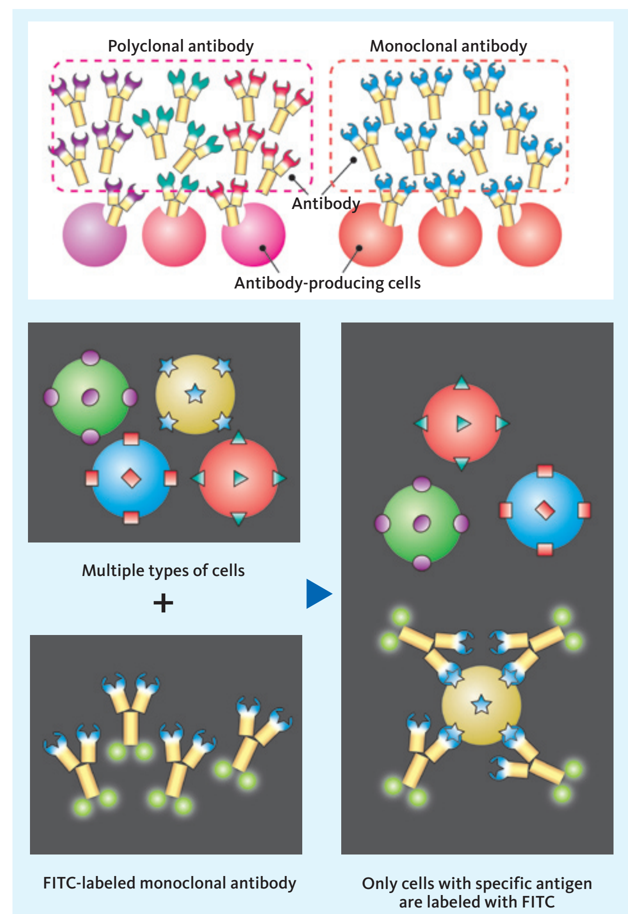
Monoclonal antibody and CD antibody

Monoclonal antibody and polyclonal antibody [3]

A single one B lymphocyte will produce one single type of antibody molecule recognising a single antigenic determinant[8]. Consequently, antibodies produced from a cell population proliferated from a specific B lymphocyte (a monoclonal population) will be identical and recognise the same determinant. Such antibodies originating from a specific B lymphocyte are called monoclonal antibodies. At the same time, in normal blood, a multitude of B lymphocyte populations exist. Even if they produce antibody molecules against the same antigen, the antigenic determinant on that antigen they recognise will vary. Such antibodies are consequently called polyclonal, originating from more than one B lymphocyte.

CD antibody

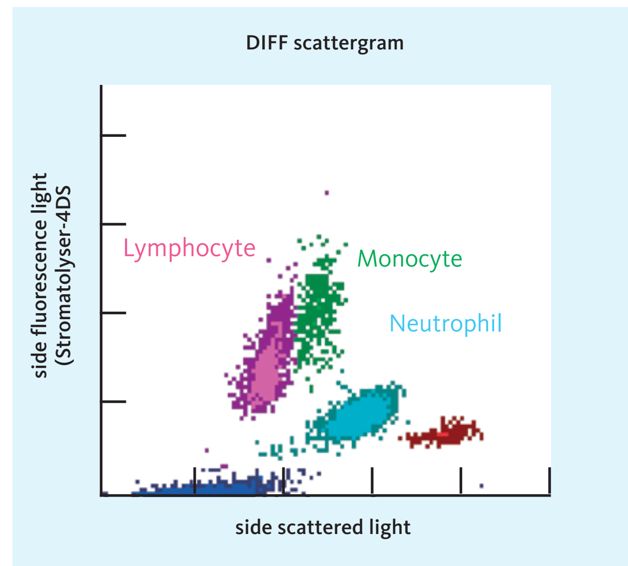
Groups of antibodies with a similar reaction pattern, and the respective marker on human cells they recognise, are grouped as so-called 'clusters of differentiation' (CD). The CD classification has been developed by the Human Leukocyte Differentiation Antigens Workshops[10] (HLDA, www.hcdm.org) and is consequentially especially used for blood cells, with an emphasis on leukocytes. In the order of approval by the HLDA, the clusters are numbered from CD1 to CD350 (as of 2008). An appropriate CD antibody reacts specifically with cells carrying the corresponding marker on the surface. Therefore, these antibodies are used to isolate or quantify the specific type of cells. CD3, CD19, CD14 and CD16b are four types of antibodies used in this study. They are antibodies to surface antigens of T lymphocytes[12], B lymphocytes, monocytes[13] and neutrophils[14] respectively.



Comparison between typing[15] by CD antibodies and leukocyte differentiation by an automated haematology analyser

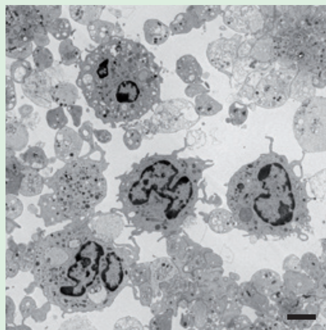
Sysmex's automated haematology analyser has the functionality to perform automated leukocyte differentiation, by staining the leukocytes with specific reagent and differentiating the leukocytes based on their appearance position on a two-dimensional leukocyte differentiation scattergram (DIFF scattergram). Side fluorescence (an indicator of staining intensities of the cell) is plotted on a vertical axis and side scatter (an indicator of complexity inside the cells) is plotted on a horizontal axis. In this study, performed on the peripheral blood of a healthy person, a mononuclear cell layer and a polynuclear cell layer[16] were separated by density gradient centrifugation[17]. From the mononuclear cell layer, negative separation[18] of CD3 positive cells (T lymphocytes), CD19 positive cells (B lymphocytes) and CD14 positive cells (monocytes) was carried out using MACS method[19]. Regarding neutrophils, MACS was not used. A polynuclear cell layer was used instead.

First, part of the sample obtained was observed with a transmission electron microscope to check the separation status. After that, each of the samples was sensitized with a respective CD antibody labeled by FITC (T lymphocytes: CD3, B lymhocytes: CD19, monocytes: CD14 and neutrophils: CD16b), and further stained with specific reagents (Stromatolyser-4DL, 4DS). Appearance positions of CD antigen positive cells on the DIFF scattergram were analysed using a flow cytometer and the staining condition was observed using a confocal laser scanning microscope.



Monocytes

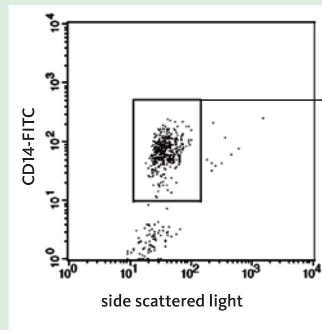
Electron microscopy image after MACS sorting.



Based on the electron microscope image after MACS sorting, it could be verified that majority of the cells are monocytes.

This sample was double stained with CD14-FITC and specific reagent, and analysis was carried out through a flow cytometer by plotting FITC fluorescence intensity and side scatter on vertical and horizontal axis respectively.

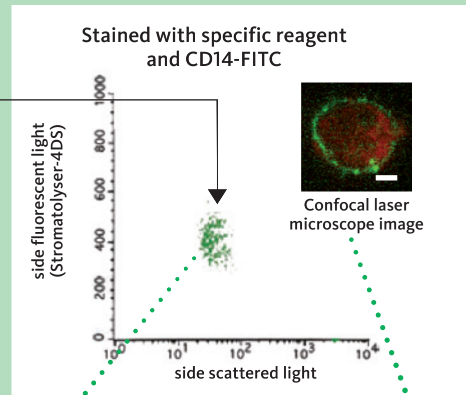
Stained with specific reagent and CD14-FITC



The cell population of the CD14 positive leukocytes strongly stained with FITC was selected and plotted on a DIFF scattergram.

It could be verified that the CD14 positive leukocytes appeared at the position of monocytes on DIFF scattergram.

DIFF scattergram reproduced with flow cytometer



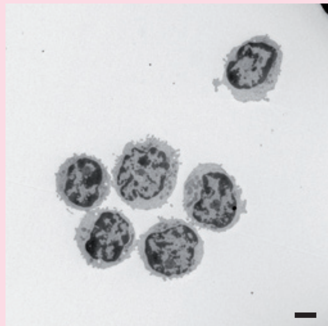
Cells appearing here were observed under a confocal laser scanning microscope. It was observed that cell surface was stained with FITC (green fluorescence) and the inside of the cell was stained with a specific reagent (red fluorescence).

Bar=2μm

Lymphocytes

B lymphocyte

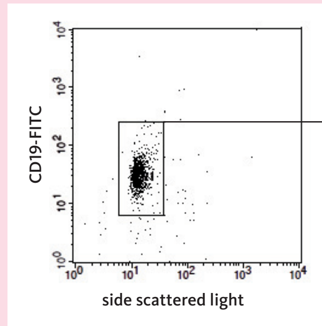
Electron microscopy image after MACS sorting



Based on the electron microscope image after MACS sorting, it could be confirmed that majority of the cells are lymphocytes.

This sample was double stained with CD19-FITC and specific reagent, and analysis was carried out through a flow cytometer by plotting FITC fluorescence intensity and side scatter on the vertical and horizontal axis respectively.

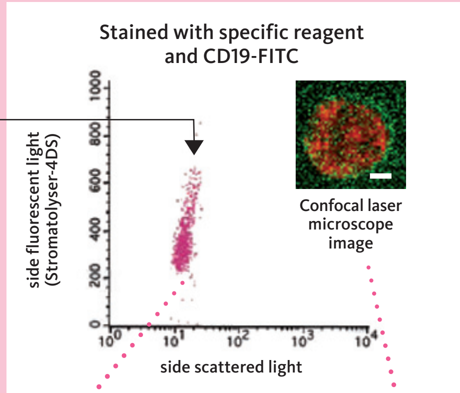
Stained with specific reagent and CD19-FITC



The cell population of the CD19 positive leukocytes strongly stained with FITC and was selected and plotted on a DIFF scattergram.

It could be verified that the CD19 positive leukocytes appeared at the position of lymphocytes on a DIFF scattergram.

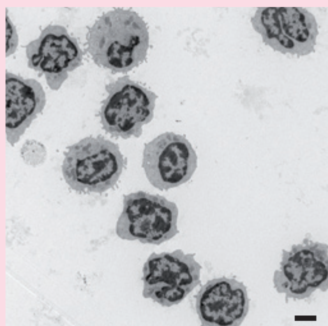
DIFF scattergram reproduced with flow cytometer



Cells appearing here were observed under a confocal laser scanning microscope. It was observed that the cell surface was stained with FITC (green fluorescence) and the inside of the cell was stained with specific reagent (red fluorescence).

T lymphocyte

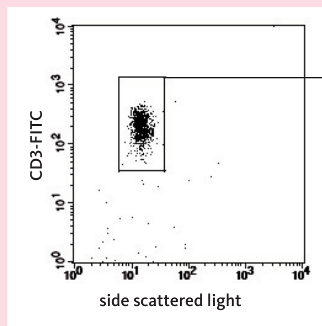
Electron microscopy image after MACS sorting.



Based on the electron microscope image after MACS sorting, it could be confirmed that majority of the cells are lymphocytes.

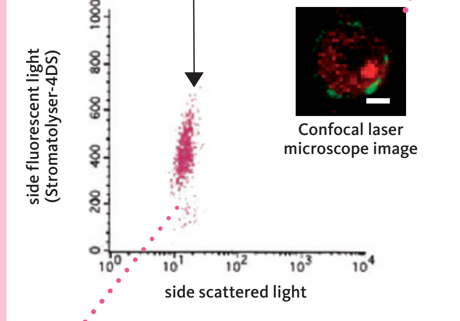
This sample was double stained with CD3-FITC and specific reagent, and analysis was carried out through flow cytometer by plotting FITC fluorescence intensity and side scatter on vertical and horizontal axis respectively.

Stained with specific reagent and CD3-FITC



The cell population of the CD3 positive leukocytes strongly stained with FITC was selected and plotted on DIFF scattergram.

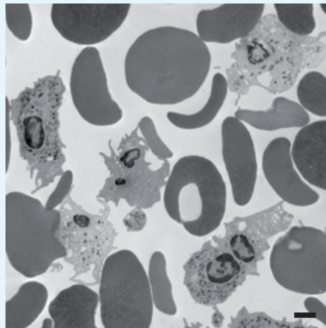
It could be verified that the CD3 positive leukocytes appeared at the position of lymphocytes on the DIFF scattergram.



Bar = 2μm

Neutrophils

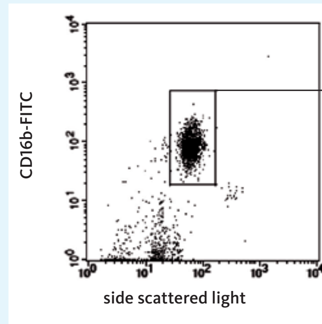
Electron microscope image after density gradient centrifugation



From the electron microscope image after density gradient centrifugation, it could be observed that the majority of the cells are polynuclear cells and erythrocytes.

This sample was double stained with CD16b-FITC and specific reagent. Analysis was carried out through a flow cytometer by plotting FITC fluorescence intensity and side scatter on vertical and horizontal axis respectively.

Stained with specific reagent and CD16b-FITC

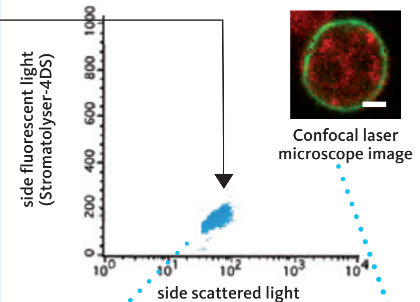


The cell population of the CD16b positive leukocytes strongly stained with FITC was selected and plotted on a DIFF scattergram.

It could be verified that the CD16b positive leukocytes appeared at the position of monocytes on DIFF scattergram.

DIFF scattergram reproduced with flow cytometer

Stained with specific reagent and CD16b-FITC



Cells appearing here were observed under a confocal laser scanning microscope. It was observed that the cell surface was stained with FITC (green fluorescence) and the inside of the cell was stained with specific reagent (red fluorescence).

Bar=2μm

Summary

In the flow cytometer analysis, CD3 and CD19 positive cells appeared at the position of lymphocytes, CD14 positive cells at the position of monocytes and CD16b positive cells at the position of neutrophils on a DIFF scattergram. Even in the observations by a confocal laser scanning microscope and a transmission electron microscope, it could be confirmed that CD-positive cells of each type express the characteristic forms of lymphocytes, monocytes and polynuclear cells respectively. Based on this validation, we could confirm that leukocyte differentiation on DIFF scattergram is in congruence with typing by CD antibodies.

Terminology

1 Labeling
Refers to attaching a fluorescent substance etc. which can act as a sign/indicator to the target molecule (an antibody in our case) in advance. Location of antigen expression can be checked with fluorescent light.

2 CD71
CD class name for transferrin receptor. It plays the role of uptaking the iron inside the cell by binding together the transferrin with high iron compatibility by membrane-bound protein that appears on the surface of the cell of an immature erythrocyte.

3 Marker
A substance that can be used as a sign or an indicator. In case of blood cells, a protein that appears on cell surface is used as a marker.

4 FITC
An abbreviation of fluorescein isothiocyanate. Fluorescein is a fluorescent dye which absorbs blue light and emits green light. FITC is a fluorescein chemical compound for binding protein to fluorescein. By mixing CD antibody attached with FITC in advance with cell population, it is possible to label only specific antigen expressing cells with green light.

5 Specifically
An antibody reacting only with the specific antigen.

6 Scattergram
A two-dimensional distribution chart plotted on the basis of two types of parameters (in DIFF scattergram, side fluorescence and side scattered light) of detected particles (cells in this case).

7 B lymphocyte
A type of lymphocyte. If it comes in contact with an antigen, it will stimulate and divide into plasmacyte to create an antibody.

8 Antigen determinant
Although an antigen refers to all the molecules that can recognize an antibody, antigen determinant refers to the domain of an antigen that can actually recognize an antibody.

9 Proliferation
Increase in the number of cells by cell division.

10 HLDA workshop
Human Leukocyte Differentiation Antigens Workshop, a series of workshops organised by the Human Cell Differentiation Molecules group with the task of characterising cell surface markers and assigning cluster of differentiation (CD) numbers to the surface molecules and the antibodies recognising them.

11 Determined quantity
Refers to deciding the quantity of a particular constituent in the sample.

12 T lymphocyte
A type of lymphocyte. Its main function it to find, attack and eliminate infected cells and adjust the immune system.

13 Monocyte
Important cell from the beginning of immunity to infection. It becomes a macrophage inside the tissue. By phagocytosis of extraneous substances like bacteria, it conveys the antigen information of extraneous substances to the lymphocyte.

14 Neutrophils
A type of granulocyte, containing granules which are positively stained with natural dyes. It performs disinfection activity through phagocytosis of bacteria, etc.

15 Typing
Deciding the type of target cells.

16 Mononuclear cell layer and polynuclear cell layer
If the whole blood sample diluted with PBS etc. is separated using a specific gravity separator gradient, a mononuclear layer containing primarily lymphocytes and monocytes, and a polynuclear layer containing neutrophils, eosinophils and basophils can be segregated at densities of d=1.077 and d=1.119, respectively.

17 Density gradient centrifugation
Method of segregation based on gravitational force using the different specific gravity of cell types. Here it is used to segregate leukocytes from a blood sample.

18 Negative separation
Method of separating only those cells which are not labeled. In MACS method, since cells labeled with magnetic beads are sorted, it is possible to obtain the target cells in the state when beads are not attached. By contrast, in positive separation target cells are labeled and picked up.

19 MACS (Magnetic Cell Sorting) method
A technique for sorting cells labeled with antibody to which magnetic beads are attached from the unlabeled cells using a powerful magnet.

Reference

[1] Kono M. et al. Reticulocyte maturation process – experimental demonstration of RET channel using anemic mice–. Sysmex Journal International. 2007; 17: 1 35–41.

[2] Fujimoto K. Principles of measurement in hematology analyzers manufactured by Sysmex Corporation. Sysmex Journal International. 1999; 9: 1 31–44

[3] Ritter M. A., Ladyman H. M. Monoclonal antibodies: production, engineering and clinical application (Postgraduate Medical Science 3). Cambridge University Press. 1995

[4] Scientific Affairs, Sysmex Corporation. The Cell Analysis Center Scientific Bulletin Part 3 Principle for measuring recticulocytes with XE-5000 and XE-2100, making use of bioimaging technology. 2007.

[5] Scientific Affairs, Sysmex Corporation. The Cell Analysis Center Scientific Bulletin Part 4 Principle for automated leukocyte differentiation with XE Family analysers, making use of bioimaging technology. 2007.

[6] Scientific Affairs, Sysmex Corporation. The Cell Analysis Center Scientific Bulletin Part 5 Action mechanism of leukocytes by special reagents for automated leukocyte differentiation (DIFF channel). 2008.

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