Principle for measuring reticulocytes with XE-5000 and XE-2100, making use of bioimaging technology

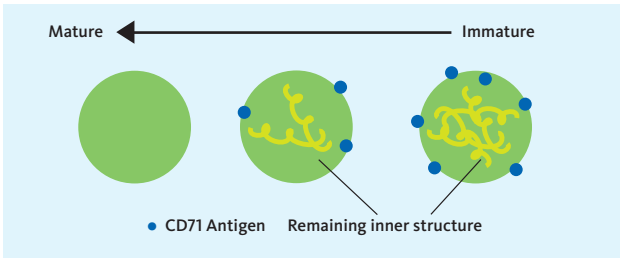
The Cell Analysis Center – Scientific Bulletin Part 3

Reticulocytes[1] are immature erythrocytes found in peripheral blood, accounting for about 1% of all erythrocytes in healthy individuals. Traditionally, reticulocytes were counted under a biological microscope after staining of the sample with new methylene blue. Development of a device using fluorescence dye (R-1000, Sysmex) has made it possible to measure reticulocytes in an automated manner. This bulletin presents the properties and morphology[2] of reticulocytes on the basis of reticulocyte scattergrams[3] (RET scattergrams) obtained with XE-5000 and XE-2100, making use of CD71 antigen.

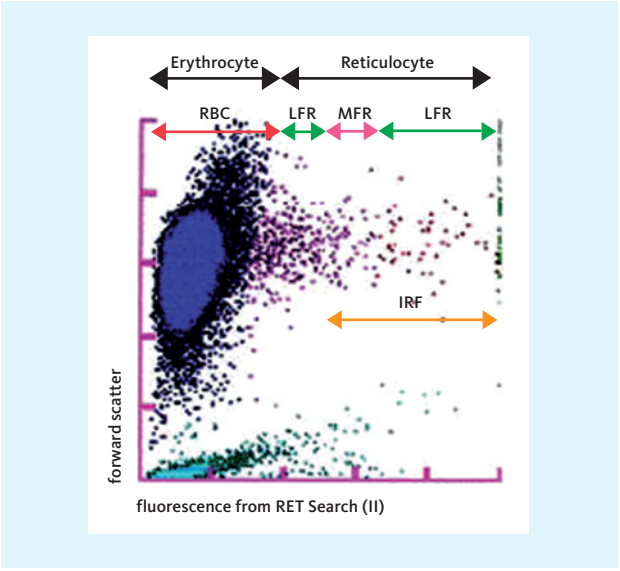
Principle for automated measurement of reticulocytes

When reticulocyte (RET) scattergrams are obtained with Sysmex automated haematology analysers, XE-5000 and XE-2100, the abundant nucleic acids[4] remaining in immature erythrocytes are stained with a fluorescent dye[5] RET Search (II). Reticulocytes are measured based on the principle of flow cytometry. The fluorescence-stained reticulocytes and erythrocytes are divided into 4 fractions by the intensity of fluorescence: HFR (High Fluorescence Reticulocytes), MFR (Medium Fluorescence Reticulocytes), LFR (Low Fluorescence Reticulocytes) and RBC (Red Blood Cell). The cells in HFR, MFR and LFR fractions[6] are counted as reticulocytes. The reticulocytes in HFR and MFR fractions are counted as IRF[7] (Immature Red Cell Fraction). The cells in RBC fraction are counted as mature erythrocytes [1].

The CD71 antigen (Transferrin Receptor)[8], a protein which receives iron for accumulation of iron in cells, is expressed[9] on the surface of reticulocytes [2]. As reticulocytes mature and adequate amounts of iron have accumulated in them, CD71 antigen is taken up by the cell and the iron is released [3]. Because of this feature, CD71 antigen is used as the surface marker[10] for reticulocytes.



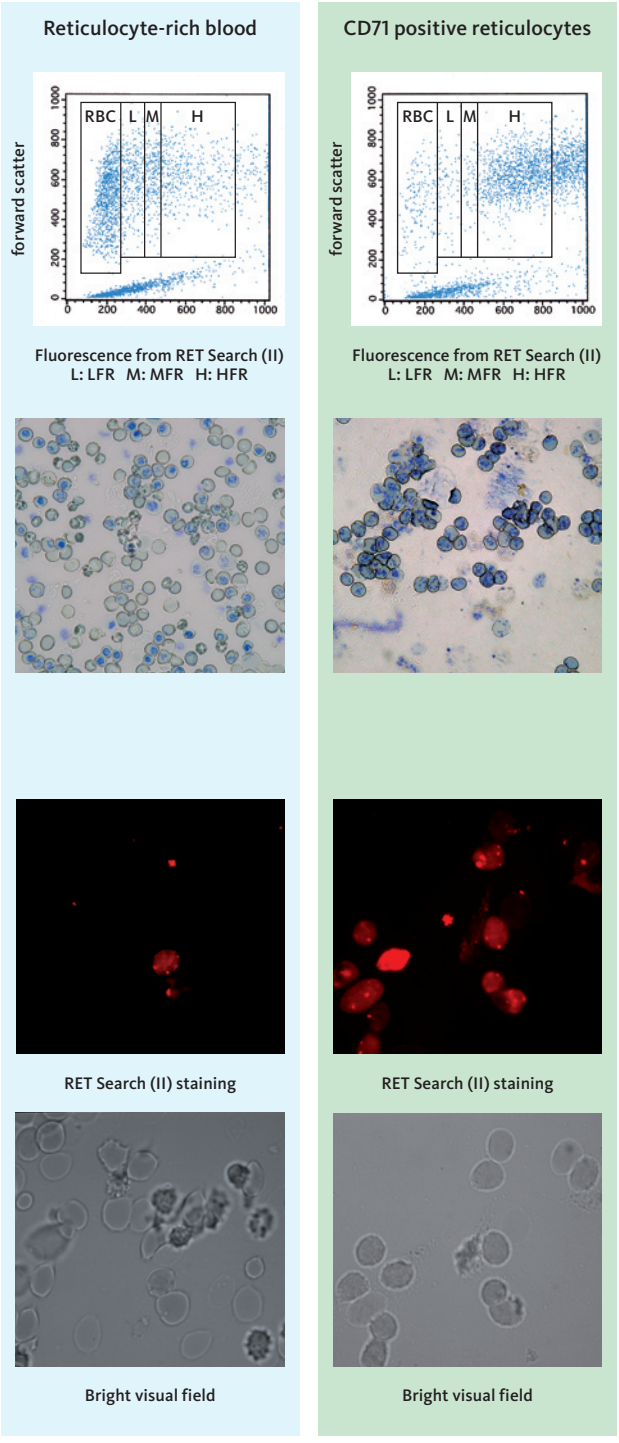
Automated haematology analyser XE-2100



RET scattergram obtained with XE-5000 and XE-2100

CD71 positive cells and reticulocytes

Reticulocyte-rich blood, obtained from peripheral blood of healthy individuals by density gradient centrifugation[11], was sensitized with magnetic beads-labeled CD71 antibody for subsequent sorting of CD71 positive reticulocytes using a powerful magnet (MACS[12]) The reticulocyte-rich blood before sorting and the CD71 positive cells after sorting were subjected to flow cytometer[13] analysis, new methylene blue[14] staining and RET Search (II) staining [4].



Flow cytometer analysis

Reticulocyte-rich blood was shown to contain cells of each of RBC, LFR, MFR and HFR fractions. On the other hand, CD71 antigen-expressed cells were sorted from reticulocyte-rich blood by MACS, showed that most of these cases were distributed in the HFR fraction on RET scattergrams.

Morphology of new methylene blue staining

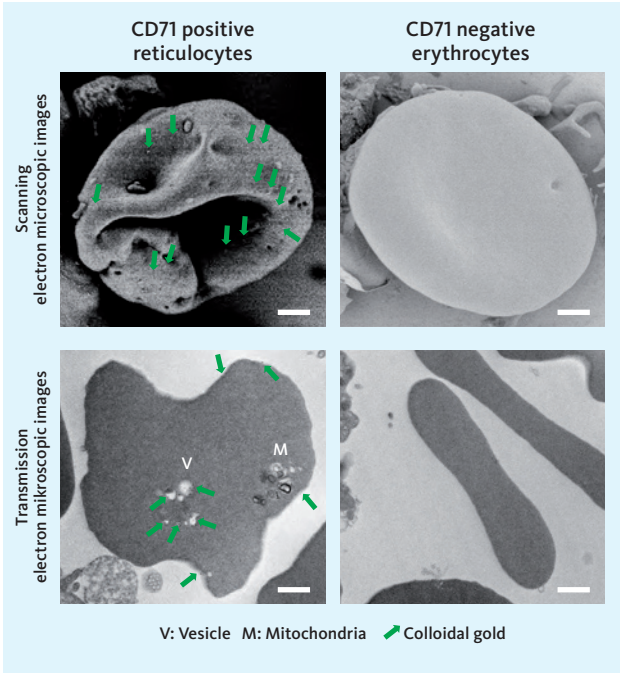
According to the Heilmeyer's classification[15], reticulocyte-rich blood was found to contain all stages of cells. The reticulocytes sorted by MACS (CD71 positive reticulocytes) contained more immature reticulocytes than class I, II and III cells according to the Heilmeyer's classification. Thus, the reticulocytes in the HFR fraction were primarily composed of CD71 antigen positive cells and more immature than Heilmeyer's class III cells.

Morphology of RET Search II staining

Reticulocytes stained with RET Search (II) were observed under a confocal laser scanning microscope[16]. Reticulocyte-rich blood contained few cells showing intense staining to RET Search (II), while reticulocytes sorted by MACS (CD71 positive reticulocytes) contained many cells intensely stained. These findings from confocal laser scanning microscopy reflected the scattergrams (obtained from flow cytometer analysis) well.

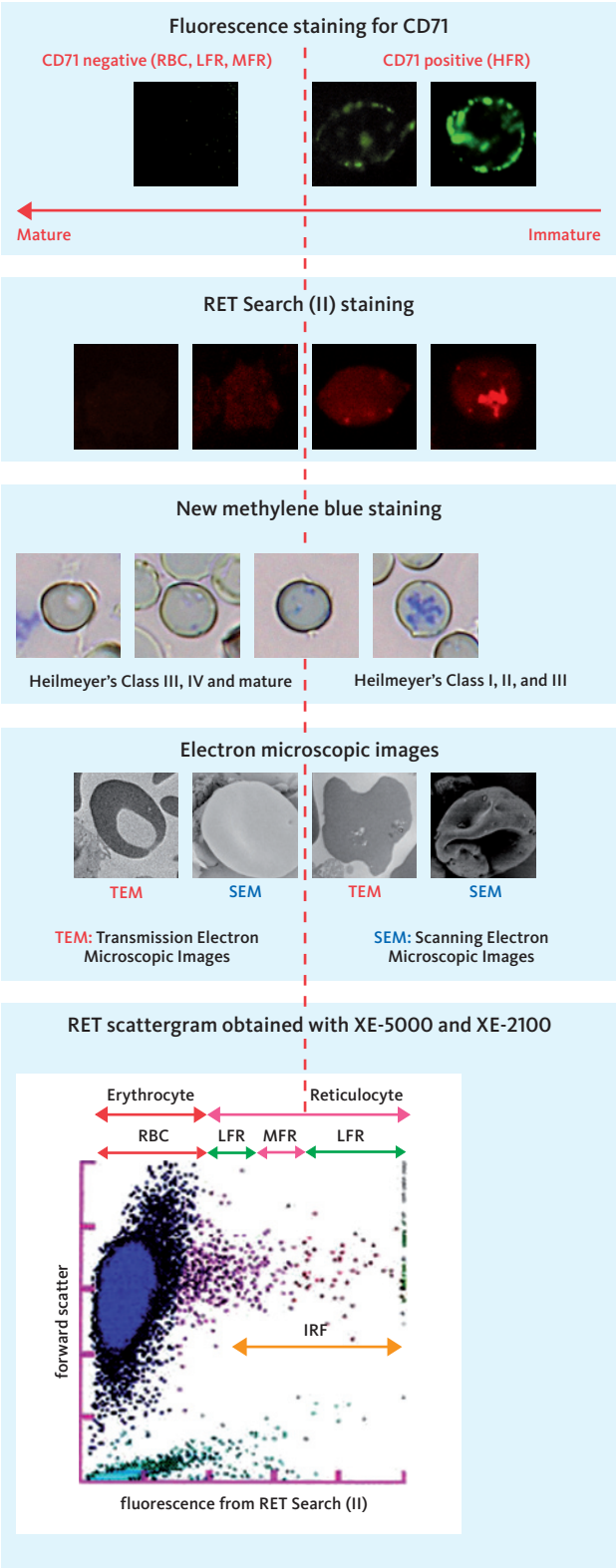
Electron microscopic images of reticulocytes

The CD71 antigen of reticulocytes was labeled with colloidal gold[17], and the cells were observed under two types of electron microscope. The scanning electron microscope[18] is designed for observation of the surface structure of cells, while the transmission electron microscope[19] is for observation of the inner structure of cells made into thin slices. Observation under a scanning electron microscope revealed the complex surface structure of CD71 positive reticulocytes labeled with colloidal gold. Under a transmission electron microscope, mitochondria[20] and vesicles[21] remaining within the cells were visible. These are morphological features of reticulocytes. CD71 negative erythrocytes were flat and poor in inner structure, resembling the mature erythrocytes in terms of morphology.



RET scattergrams obtained with XE-5000 and XE-2100 compared with cell imaging

The cells showing intense staining to RET Search (II) and shown as HFR fraction on the RET scattergram, obtained with XE-5000 and XE-2100, were CD71 positive reticulocytes. These cells were rich in inner structure and assumed a typical morphology of reticulocytes. The reticulocytes in MFR and LFR fractions showed weak staining to RET Search (II) and most of them were CD71 antigen negative, assuming a form akin to mature erythrocytes under an electron microscope. This study revealed the relationship between reticulocyte morphology and CD71 expression. These morphological findings allowed us to confirm that the RET scattergrams obtained with XE-5000 and XE-2100 reflect the reticulocytes maturation well.



Terminology

1 Reticulocyte
Immature erythrocytes just released from bone marrow into blood vessels. About 1% of all erythrocytes are reticulocytes in healthy individuals.

2 Morphology
In this bulletin, it indicates the forms of cells observed under electron microscope, confocal laser scanning microscope and biological microscope.

3 Scattergram
Graphic representation of optical information of cells collected with a flow cytometer. Physical and chemical properties of cells are presented.

4 Nucleic acid
A macromolecule found in organisms. Can be divided into DNA and RNA. DNA is associated with genetic information in nuclei, while RNA is involved in expression of genetic information.

5 Fluorescent dye
A collective term for substances which, after absorbing electromagnetic radiation such as light, themselves emit radiation, usually of a longer wavelength than that of the absorbed radiation (e.g. absorbing ultraviolet light and emitting visible light). If a fluorescent dye is bound to particles or substances, it allows accurate location, observation and measurement of potential changes in the target.

6 Fraction
Cells are classified under various conditions preset for a given device. In the present study, the cells were classified according to the intensity of scatter and fluorescence as measured with a flow cytometer.

7 IRF
Of the fractions HFR, MFR and LFR of reticulocytes measured with XE-5000 and XE-2100, the percentage of particularly immature cells (HFR + MFR) is called IFR. This serves as an indicator of hemopoietic capability of bone marrow.

8 CD71 antigen, transferrin receptor
Membrane-bound proteins which bind to transferrin and receive iron.

9 Expression
Synthesis of proteins based on genetic information.

10 Surface marker
Proteins specifically expressed on the surface of target cells. They are called 'markers' since they are used to identify target cells.

11 Density gradient centrifugation
Centrifugation under gravitational force, making use of the difference in specific gravity depending on the type and maturity level of cells.

12 MACS
A technique for sorting cells with a powerful magnet after attachment of magnetic beads to the surface of target cells in a sample, making use of antigen-antibody reactions.

13 Flow cytometer
Small particles such as cells are dispersed in a fluid, and the fluid is flowed through a small nozzle for optical analysis of individual particles.

14 New methylene blue
A dye used to count reticulocytes under a normal microscope.

15 Heilmeyer's classification
The maturity level of reticulocytes can be assessed by their intensities to new methylene blue staining. In 1931, Heilmeyer proposed classification of the stages of reticulocyte maturation on the basis of staining intensities. This classification is often used for assessment of the maturity level of reticulocytes even at present.

16 Confocal laser scanning microscope
A microscope with laser serving as a light course, capable of achieving high spatial resolution not possible with a fluorescence microscope. Also capable of providing sectional images of cells stained with fluorescence.

17 Colloidal gold
Gold particles (1–40nm) used for immunoelectron microscopy. Gold with a high electron density is visible as black particles under a transmission electron microscope and as white particles under a scanning electron microscope.

18 Scanning electron microscope
A microscope allowing observation of the ultramicrostructure of the cell surface. A special metallic film is formed on the cell surface, and electron beams are applied to it for observation of the cell surface.

19 Transmission electron microscope
A microscope allowing observation of the microstructure inside cells. The cells are made into thin slices (70 nm) and electron beams are applied to them for visualization of electrons.

20 Mitochondria
An organelle serving as the place of oxygen respiration and energy production.

21 Vesicle
An organelle covered with lipid membrane and partitioned from the cytoplasm. Involved in storage, transport, production, disposal of waste, etc.

Reference

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[6] Scientific Affairs, Sysmex Corporation. The Cell Analysis Center Scientific Bulletin Part 2 Electron microscopy of reticulocytes after sorting with magnetic beads. 2007.

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