TECHNICAL REPORT

SERIES 9

Reagent Characteristics in the XE-2100 NRBC Channel

Takashi SAKATA

R&D, Sysmex Corporation,
4-4-4 Takatsukadai, Nishi-ku, Kobe 651-2271, Japan.

Key Words

Flow Cytometry (FCM), Semiconductor Laser, NRBC, Polymethine Dye

INTRODUCTION

The measurement of nucleated red blood cell (NRBCs) not only provides important information for determining treatment in cases such as thalassemia, etc. but can also give useful information in diagnosing chronic myeloproliferative disorders such as myelofibrosis and chronic myeloid leukemia[1-3]. However, in the conventional (manual microscopic) method, the number of cells counted is usually as few as 100 cells, causing problems in accuracy and precision[4]. In addition, with a typical routine automated hematology analyzer, it is difficult to completely distinguish between NRBC and WBC, and NRBC tends to interfere in the WBC count. To resolve these problems, NRBC counting methods using various FCM have been developed[5], but few satisfactory results have been obtained.

The XE-2100 has made it possible to accurately count NRBC by using a newly developed NRBC measurment reagent, "STROMATOLYSER-NR" and the FCM detector using a red semiconductor laser.

In this report, the NRBC measurement reagent "STROMATOLYSER-NR" and the measurement principle of the XE-2100's NRBC channel will be described. In addition, a new NRBC reference method using FCM will be introduced.

NRBC MEASUREMENT REAGENT STROMATOLYSER-NR

The NRBC measurement reagent "STROMATOLYSER-NR" comprises a stain containing polymethine dyes, and an acid-hyposmotic diluent containing surface active agents. The composition and mechanism of the reagent will be described as follows.

Stain solution (Polymethine Dyes)

For cell staining, various dyes are used depending on the objects to be stained;but generally speaking, in order for a dye to stain a cell and measure it using FCM, the following characteristics are required:

1) Fluorescent capabilities: The ability to absorb beams of light (e.g. red semiconductor laser, λ = 633 nm for XE-2100).
2) To have cell membrane permeability and specifically associate with the desired cell component.
3) To fluoresce only when associated with the targeted cell component.
4) Chemically stable.
5) Low toxicity.

We have developed a polymethine dye that satisfies these conditions and that can be excited with a semiconductor laser (633 nm). Polymethine is typically used as the functional dyes that supports latest industrial technolo-

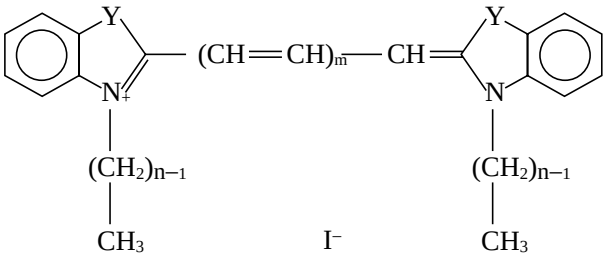

Fig. 1 *General structural formula of polymethine dyes*

gies such as near-infrared absorbing dyes, dye lasers, LB membranes, and color photo film sensitizers[6]. In the cytometry field, polymethine dyes are known for measuring cell membrane potential[7-9], but they have rarely been used for staining in hematologic or pathological tests. However, polymethine dyes following provide the following properties that are well-suited for cell staining:

1) Polymethine dyes are structurally characterized by the binding of a heterocycle (cyclic compound containing nitrogen, sulfur, and oxygen) and a methine chain (-CH2=). These heterocyclic structures are known as fluorescent chromophores, and many dyes having these structures fluoresce (***Fig. 1***).
   Polymethine dyes are able to have the wavelength of the light absorbed theoretically designed by changing the kind of heterocyclic nucleus, binding side chain, length of the methine chain. For example, as the number of "m" for the methine chain -(-CH=CH-)$_m$- to which the heterocyclic nucleus is joined is increases by 1, the absorption wavelength of the dye shifts toward the longer-wavelength side by about 100 nm. In this way, polymethine dyes have an advantage in that dyes of desired absorption wavelength are developed comparatively easily by changing the structure of dye molecules[6].
2) It is comparatively easy to control the cell membrane and nuclear membrane permeability of polymethine dye. As well as its affinity to cell components by changing molecular structure. In general, polymethine dyes have positive charges in the molecular structure, and are easily linked by nucleic acids and anion. It is also possible to be bounded by intercalation in double helix of DNA[10].
   Cell membrane permeability will be described in the discussion of the diluent.
3) Polymethine dyes existing in aqueous solution provide unique features in that they do not fluoresce because the light energy absorbed is converted to heat energy by intramolecular diffusion motion, but when bound to cell membranes or nucleic acids, they increase light absorption and intensify fluorescence because intramolecular diffusion motion is interfered with[11, 12].
4) Polymethine dyes are rather chemically unstable substances, and are easily decomposed by water, light, and oxygen. However, in STROMATOLYSER-NR, the dyes are stabilized by using ethylene glycol (a nonaqueous solvent system) as a storage solvent. On the other hand, the chemical instablity in turn becomes an advantage, because it is able to be decomposed when discharged into the environment as waste.
5) It is reported that polymethine dyes are safe, with far less mutagenicity as compared to specific fluorescent dyes binding to nucleic acid (such as ethidium bromide)[13].

In these ways, polymethine dyes provide excellent properties as dyes. In the XE-2100, by developing and using optimum polymethine dyes not only for the NRBC channel, but also the 4 DIFF and RET channels, a wide variety of normal and abnormal cells can be classified and counted[14].

Diluent (Hemolytic agent)

When WBC measurement is carried out by an automated hematology analyzer, the anticoagulated peripheral blood must be treated with a suitable diluent.
The diluent provides several functions as shown below.

1) Dilution of whole blood
   If whole blood is measured without dilution, the number of cells is far too numerous to be counted by the detector. The sample therefore must be diluted to a suitable concentration so that cells pass through the detector one by one. In general, to measure WBC, the whole blood is diluted anywhere from10 times to hundreds of times.
2) Lysis of RBC
   RBC number more than 1,000 times that of WBC in the peripheral blood, and will hinder NRBC measurement. In order to eliminate the influence of RBC, the RBC must be selectively lysed.
3) Treatment of cells
   In order to stain specifically and distinguish the cells to be measured, physiochemical conditions such as pH and ion intensity must be set to optimum conditions in accordance with the cell to be measured. In STROMATOLYSER-NR, an acidic hypotonic solution is used.
4) Adjustment of dye permeability
   Polymethine dyes provide comparatively good cell membrane permeability. In STROMATOLYSER-NR, a dye permeability accelerator is added to complete staining in a shorter time (A littel over ten seconds).

Diluent is required to provide various functions such as lysing of RBC, adjustment of staining capability and promotion of dye permeability, while maintaining the form of other blood cells.
This section describes the lysis of RBC in detail.
There are four primary methods of lysing RBC: 1) change of osmotic pressure, 2) lowering of surface activity in the suspension solution, 3) decomposition of membrane constituents, and 4) strong physicochemical interaction between membrane substances and other substances[15]. A variety of RBC lysing methods have been reported[16-21].
Hemolytic agents used in automated hematology analyzers

Table 1 *Hemolytic agent*

Main constituents	Advantages	Disadvantages	Remarks
Hypotonic solution	• Little damage to leukocytes. • Able to maintain cell form and features close to *in vivo*. • RBC are able to be selectively lysed by adjusting the pH of the solution to acidic.	• RBC are lysed; but since erythrocytic membranes remain as ghosts, it is not well suited for electric resistance measurement method.	• Popularly used for optical measurement method.
Cationic surface active agents	• Extremely quick hemolysis reactions. • Comparatively small RBC ghosts.	• Damage to cells is comparatively large and cell form is likely to chang.	• Popularly used for electric resistance measurement method.
Nonionic surface active agents	• Extremely quick hemolysis reactions. • RBC ghosts can be completely dissolved.	• Damage to cells is comparatively small.	• Applied as an intermediate method between above two methods.

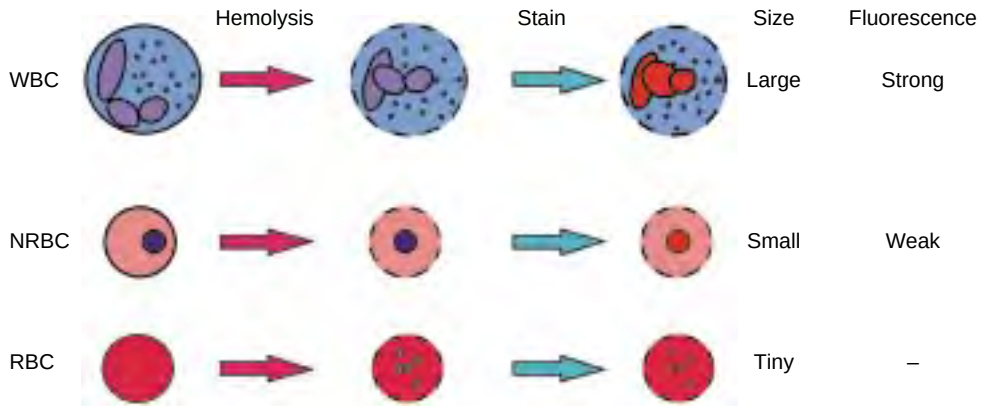

Fig. 2 *NRBC channel measurement principle*

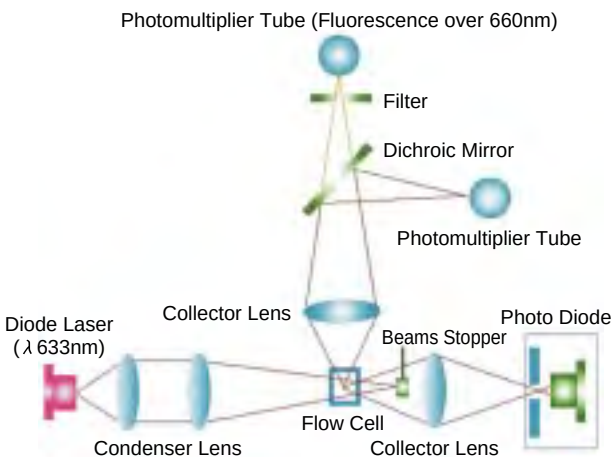

Fig. 3 *Block diagram of optical system*

fall into the following three general categories (***Table 1***), and an optimum hemolytic agent is used depending on the cell to be measured.
Because in STROMATOLYSER-NR diluent RBC must be nearly completely dissolved and the staining capabilities differentiated between WBC and NRBC, the acidic hypotonic solution containing cationic surface active agent is used.

NRBC measurement principle (*Figs. 2 and 3*)

Mixing of blood with the NRBC measurement reagent "STROMATOLYSER-NR" diluent dissolves the membrane lipid constituents of RBC and forms osculums in the cell membrane. The RBC effuses its hemoglobin, and shrinks to a cell membrane only, becoming optically transparent. NRBC generates pores in the cell membrane in the same mechanism of action, effuses hemoglobin and at the same time, accelerates the dye permeability. The WBC generates pores in the cell membrane as in the case

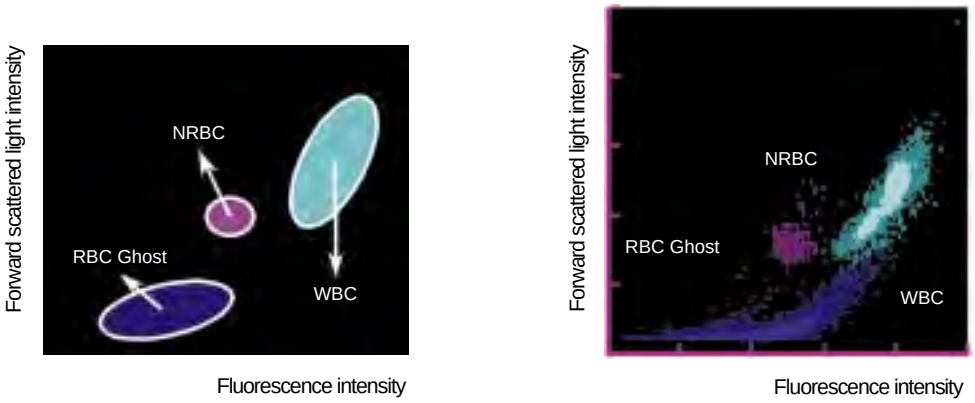

Fig. 4 *NRBC scattergram*

of NRBC and has the dye permeability accelerated. Organelles of WBC, however, are not effused but remain in the cell. Allowing the "STROMATOLYSER-NR" stain to act on under this condition stains nucleic acids, organelles, nuclei inside the WBC with the polymethine dyes. On the other hand, the nuclei of NRBCs are difficulty binding dyes and stain weakly because the nuclear chromatin condenses.
The stained cells are directed into the flow cell and illuminated with the 633nm of red laser beam. The forward scattered light intensity and side fluorescence intensity produced from the cells are detected. The information of side fluorescence intensity is plotted on the abscissa and the forward scattered light intensity on the ordinate in the two-dimensional scattergram.

1) Since RBC turn to become cell membranes (ghosts) by the action of the diluent (lysis), light is minimally scattered; therefore, the forward scattered light intensity is low. Since RBC membranes are scarcely stained, they appear in the vicinity of origin at the lower left where the side fluorescence light intensity is low, that is, in the ghost area.
2) Since WBC maintain their form despite pores are generated in cell membranes by the action of the diluent, they scatter light strongly. WBCs are also strongly stained with polymethine dyes; thus the forward scattered light intensity is high, and these cells appear in the WBC area at the upper right (or middle) where fluorescence intensity is large.
3) Because NRBCs become nearly bare nuclei by the action of the diluent, the forward scattered light intensity is low. Since the cells are weakly stained with polymethine dyes, they appear in the NRBC area located in between the ghost area (with low fluorescence intensity) and the WBC area.

As described above, because NRBC and WBC can be clearly distinguished from each other by the difference in both of the forward scattered light intensity and the fluorescence intensity, NRBCs can be accurately classified and counted (***Fig. 4***). The NRBCs counted in this way are indicated as NRBC% (/100WBC) and NRBC# (/μL) in conformity to the evaluation standard NCCLS H20-A[22] when 20 or more counts/μL appear.

NRBC REFERENCE METHOD

In general, to evaluate the WBC differential performance of the hematology analyzer, NCCLS H20-A, which is a standard method using a microscope, is used. The manual method is an excellent method that can evaluate various types of blood cells all at once, but it is known for its statistical problems due to the small number of cells counted. Also, individual differences in judgment of the technologists performing the count is an issue; the manual differential is not always an accurate or objective method. Our company has devised a flow cytometry-based reference method of evaluating the performance of the hematology analyzer[23]. Recently, an NRBC reference method using FCM has been newly devised for evaluating NRBC counting performance of the hematology analyzer[24] (***Figs. 5 and 6***). This method stains each of the cells using the monoclonal antibody (FITC labeled CD45 antibody) for recognizing the WBC common antigen and nucleic acid staining dyes (propidium iodide), and specifically detects and counts NRBC. Consequently, WBC, NRBC, and erythrocytic ghosts can be clearly identified, and NRBC% is able to be accurately counted. This method measures 10,000 cells (as compared to the WBC-100 count of the manual method) and provides superior accuracy.
The following figures show the evaluation results of NRBC measurement performance of XE-2100 and a measurement example with the previously described method used as a reference.

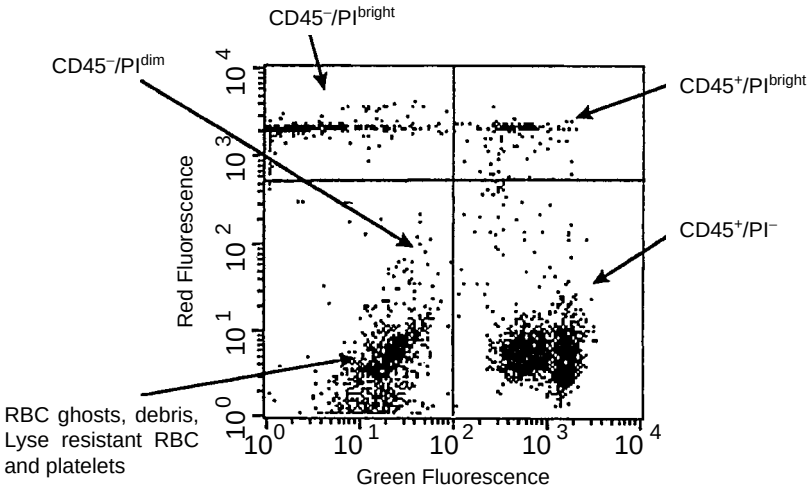

Fig. 5 *Example of NRBC measurement by FCM*

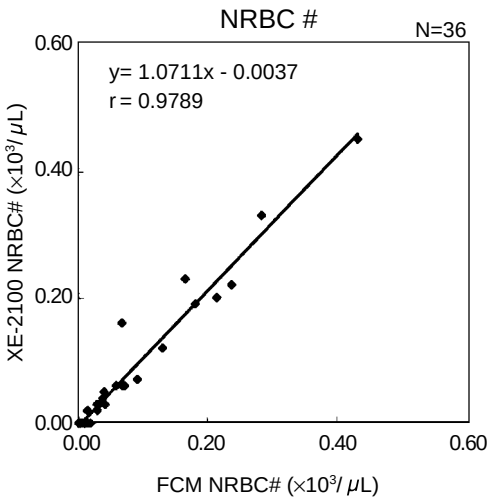

Fig. 6 *Correlation diagram of NRBC counts between FCM and XE-2100*

CONCLUSION

The technological capabilities of multi-parameter hematology analyzers are continually expanding. Instruments are now measuring and differentiating cell types that were previously thought to be impossible to distinguish "electronically." In this report, the XE-2100 NRBC counting principle has been described with emphasis placed on the STROMATOLYSER-NR reagent capabilities. This information may help to confirm the accuracy of the XE-2100 NRBC count and its subsequent clinical utility.

We believe the automated blood cell counter will continue to bring tremendous clinical advances to healthcare in the future, and it is our everlasting assignment to explain and present our new technology to you. We hope this will allow you to understand and develop confidence in our ability to count all types of cells-including, in the future, cells now considered "impossible" to count.

References

1) *Schwartz FO, Stanbury F. : Significance of nucleated red blood cells in peripheral blood. JAMA, 154 : 1339-1340, 1954.*

2) *Weick JK, et al. : Leukoerythroblastosis : diagnostic and prognostic significance. Mayo Clin Proc, 49 : 110-113, 1974.*

3) *Nelson D A, Morris MW: Basic examination of blood. In : Henry JB editor. Clinical diagnosis and Management by laboratory method, 18th edition. Philadelphia : W. B. Saunders Company, P 589-590, 1991.*

4) *Koepk JA, et al. : A Critical evaluation of the manual/visual differential leukocyte counting method. Blood Cells, 11 : 173-186, 1985.*

5) *Terstappen LW, et al. : Discriminating between damaged and intact cells in fixed flowcytometric samples. Cytometry, 9 : 548-556, 1998.*

6) *Nippon Kankoh-Shisiko Kenkyusho Co., Ltd. : editor. Kankoh-Shisikiso, 1st edition. Tokyo : Sangyo-Tosyo, 1997.*

7) *Waggoner AS : Optical probes of membrane potential. J Membrane Bio, 27 : 317-334, 1976.*

8) *Waggoner AS : Dye indicators of membrane potential. Ann Rev Biophys Bioeng , 8 : 47-68, 1979.*

9) *Waggoner AS : Fluorescent probes for cytometry, Flow Cytometry and Sorting (2nd Edition), pp. 209-225, 1990.*

10) *Berman HM, Young PR : The Interaction of intercalating drugs with nucleic acids. Ann Rev Biophys Bioeng, 10 : 87-114, 1981.*

11) *Lee LG, et al. : Thiazole Orange : A new dye for reticulocyte analysis. Cytometry, 7 : 508-517, 1986.*

12) *Rye HS, et al. : Stable fluorescent complexes of double-stranded DNA with bis-intercalating asymmetric cyanine dyes : properties and applications. Nucleic Acids Research, 20 : 2803-2812, 1992.*

13) *Singer VL, et al. :Comparison of SYBR Green I nycleic acid gel stain mutagenicity and ethdium bromide mutagenicity in the Salmonella / mammalian microsome reverse mutation assay Ames test. Mutation Research, 439 : 37-47, 1999.*

14) *Matsumoto H: The technology of reagents in the automated hematology analyzer Sysmsex XE-2100-red fluorescence reaction.*

Sysmex J Int, 9 : 179-184, 1999.

15) *Kondo T : Hemolysis by surface-active agents. Hyommen, 5 : 630-635, 1967.*
16) *Dodge JT, et al. : The preparation and chemical characteristics of hemoglobin-free ghosts of human erythrocytes. Archives Biochem Biophys, 100 : 131-139, 1963.*
17) *Thron CD. : Hemolysis by holothurin A, digitonin, and quillaia saponin: Estimates of the required cellular lysin uptakes and free lysin concentration, 145 : 194-202, 1964.*
18) *Kondo T, Tomizawa M : Hemolysis by nonionic surface-active agents. J Pharm Sci, 57 : 1246-1248, 1968.*
19) *Tmonizawa M, Kondo T : Mechanism of hemolysis by anionic suragface-active agents. Kolloid-Z u Z Polymere, 246 : 694-699, 1971.*
20) *Kondo T, Tomizawa M : Hemolytic action of surface-active electrolytes. J Colloid Interface Sci, 21 : 224-228, 1966.*
21) *Helenius A, Simons K: Solubilization of membrane by detergents. Biochim Biophys Acta, 415 : 29-79, 1975.*
22) *National Committee for Clinical Laboratory Standards (NCCLS) : NCCLS H-20A*
23) *Lebeck LK, et al. : White blood five-part subpopulation estimations : A flow cytometric based reference method. Sysmex J Int, 5 : 77-84, 1995.*
24) *Tsuji T, et al. : New rapid flow cytometric method for the enumaeration of nucleated red blood cells. Cytometry, 37 : 291-301, 1999.*