Reference Method for Platelet Enumeration

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The main principle of enumerating platelets (PLT) in automated hematology analyzer is electric resistance system. However, there have been an increasing number of instruments that can enumerate PLT by optical system. The use of a flow cytometry (FCM) employing monoclonal antibody has been under study for the enumeration of PLT in recent years. At its meeting held in April 2000, the International Society for Laboratory Hematology (ISLH) decided on a protocol to study the possibility of using, as reference method, a monoclonal antibody-employed FCM of enumerating PLT. In our present study, we enumerated PLT by the monoclonal antibody-employed method proposed by ISLH, and compared the result with the PLT count obtained from XE-2100, an automated hematology analyzer, employing electric resistance system, and another PLT count obtained by optical system, and the results of the comparison are reported here.

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Key Words Platelets (PLT), Reference Method, International Society for Laboratory Hematology (ISLH), Flow Cytometry (FCM), Monoclonal Antibody, Hematology Analyzer, Platelets

1st Report: Report of enumeration data on samples from normal subjects

INTRODUCTION

The main principle of enumerating platelets (PLT) in automated hematology analyzer is electric resistance system. However, there have been an increasing number of instruments that enumerates PLT by optical system. Reference methods for enumeration of white blood cell (WBC) and red blood cell (RBC) have been established by the International Council for Standardization in Haematology (ICSH)[1]. As for enumeration of PLT, there has been a delay in the establishment of such a reference method. In our company, we developed a semi-automatic PLT enumerating instrument with the use of electric resistance sheath flow system (SSF)[2], and we have been using this analyzer until the establishment of an official reference method.

The use of a flow cytometry (FCM) employing monoclonal antibody has been under study for the enumeration of PLT in recent years[3-5]. At our company, too, we used CD42a, and announced the results of our use in 1996[6]. With such circumstances in the background, the International Society for Laboratory Hematology (ISLH) held in April 2000 decided on a protocol to study the possibility of using, as reference method, a monoclonal antibody-employed method for the enumeration of PLT.

In our present study, we enumerated PLT using the monoclonal antibody-employed method proposed by the ISLH. The result was compared with the PLT count (hereinafter referred to as "impedance platelet (PLT-I)") obtained from the XE-2100, an automated hematology analyzer employing electric resistance system, and another PLT count (hereinafter referred to as "optical platelet (PLT-O)") obtained by the optical system. The results of the comparison are reported here.

SAMPLES

K_2EDTA samples from healthy normal subjects: n=20; samples from patients: n=25 (Tube used for blood collection: Venoject II, TERUMO).

Patient samples obtained from thrombocytopenia were used, excluding the following samples: idiopathic thrombocytopenic purpura (ITP), mean corpusular volume (MCV)<75fL, schistocyte, hemagglutination, within 5 days after blood transfusion, PLT aggregation, hemolysis, etc.

Enumeration was made within 6 hours after blood collection, and samples were kept at room temperature until the time of enumeration.

ENUMERATING INSTRUMENTS

FACSCalibur (Becton Dickinson), and the XE-2100 (Standard counter at Scientific Division, Sysmex Corporation)

REAGENTS USED

Monoclonal antibody: CD41-FITC (Beckman Coulter); CD61-FITC (Becton Dickinson); Simultest control γ_1/γ_2a (negative control, Becton Dickinson)

Diluent: phosphate buffered saline (PBS) containing 0.1% bovine serum albumin (BSA).

DETAILS OF ENUMERATING METHOD

5 µL of blood collected by K_2EDTA (Venoject II, TERUMO), 5 µL of monoclonal antibody (CD41 or CD61) for PLT enumeration, and 100 µL of PBS (0.1% BSA) are pipetted into a tube for FCM (Falcon No. 2054, Becton Dickinson).
After 8 slow inversions, the mixture is incubated for 15 minutes in a dark place at room temperature.
750 µL of PBS (0.1% BSA) and 10 µL of incubated sample are pipetted into a separate tube for FCM. The mixture is subjected to 8 slow inversions, and then enumeration is done with FACSCalibur, followed by analysis of PLT/ RBC (***Fig. 1***).
As a control, Fonio method (1,000 count) was performed. As for PLT count in FCM and Fonio method, enumeration was done using RBC enumerating data obtained from the XE-2100. RBC count and WBC count obtained from the XE-2100 were calibrated by ICSH's reference method[1)], and the PLT count was calibrated by the aforementioned SSF[2)]. After these calibrations, the sample was subjected to enumeration.

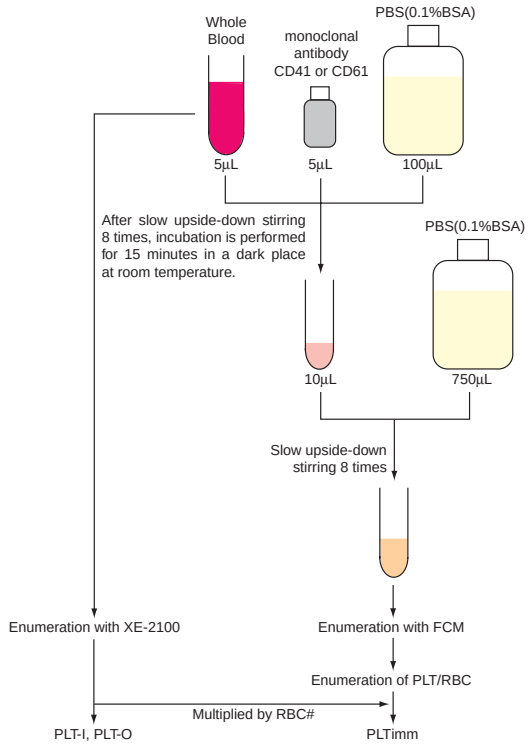


Fig. 1 *Protocol for enumeration*

RESULTS

Examination of final total dilution

Using samples from 5 normal healthy subjects, the final total dilution examined was up to 500–3,000-fold dilution. The results showed that the higher the total dilution was, the lower was the simultaneous-pass effect (***Fig. 2***). The simultaneous-pass effect was large at 500-fold dilution. Thereafter, however, the simultaneous-pass effect sharply decreased. Therefore, it is desirable to prepare samples at 1,000-fold or greater dilutions. However, the higher the total dilution is, the lower becomes the number of events per unit time becomes, leading to longer FCM enumeration time per sample. As a result, there is a high possibility that the numerical value of FCM may contain debris such as foam. Consequently, in our present study, the final total dilution was set at 1,500-fold dilution.

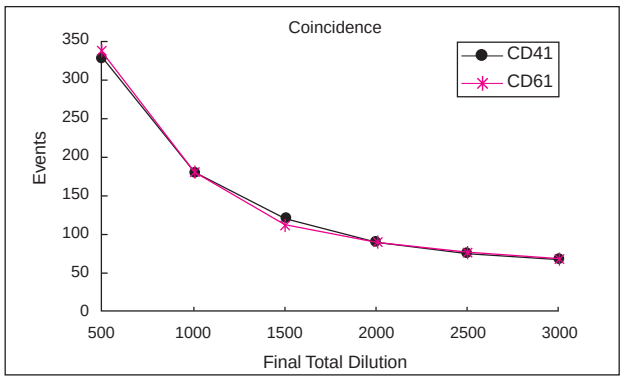


Fig. 2 *Examination of final total dilution*

Correlation between Fonio method and each enumerating method

Correlation with Fonio method was studied in samples from normal subjects and in samples from thrombocytopenia. The FCM result with the use of CD41 was a regression equation of y=1.023x + 12.543 with a correlation coefficient of r=0.875. With the use of CD61, the regression equation of y=0.991x + 18.269 with a correlation coefficient of r=0.861. Similarly, when the XE-2100 was used, the comparison with PLT-I data was a regression equation of y=1.122x + 2.401 with a correlation coefficient of r=0.874. With PLT-O data, the regression equation was y=1.066x + 8.553 with a correlation coefficient of r=0.863 (***Fig. 3***). Each enumerating method showed a similar tendency, and when reproducibility in the visual examination is taken into consideration, there was a good correlation.

Correlation between FCM and XE-2100

Correlation between FCM and the XE-2100 was studied in samples from normal subjects and samples from thrombocytopenia. When CD41 and PLT-I were used, the regression equation was y=0.909x + 9.470 with a correlation coefficient of r=0.998. When PLT-O was used, the regression equation was y=0.943x + 6.169 with a correlation coefficient of r=0.996. Similarly, when CD61 and PLT-I were used, the regression equation was y=0.892x + 12.961 with a correlation coefficient of r=0.995. With PLT-O, the regression equation was y=0.928x + 9.300 with a correlation coefficient of r=0.996 (***Fig. 4***). Thus, the correlation coefficient was 0.99 or greater in all cases, indicating very good correlation.

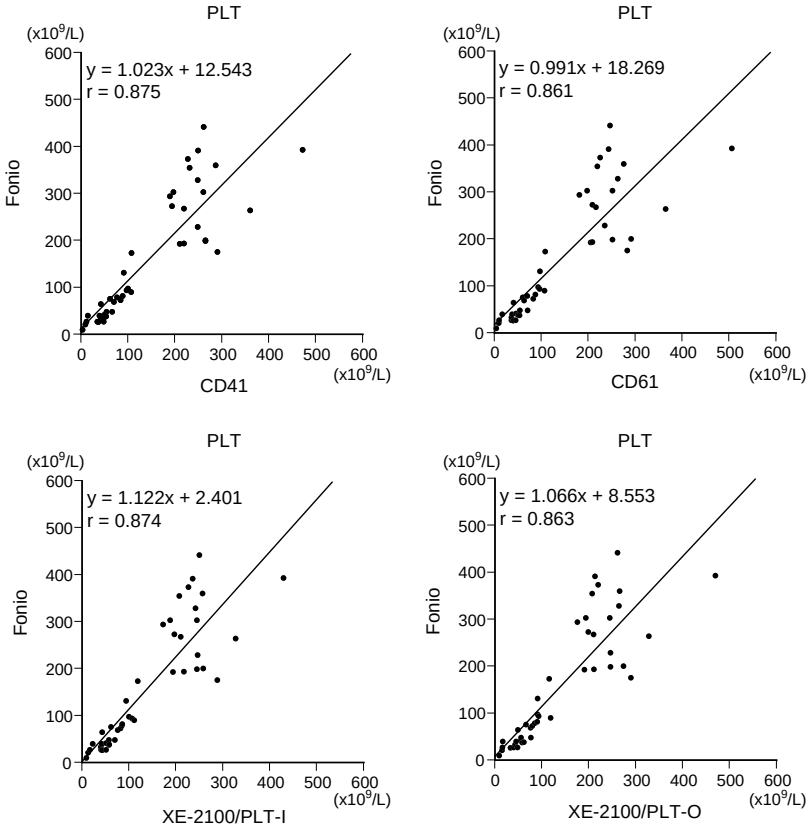


Fig. 3 *Correlation with Fonio method*

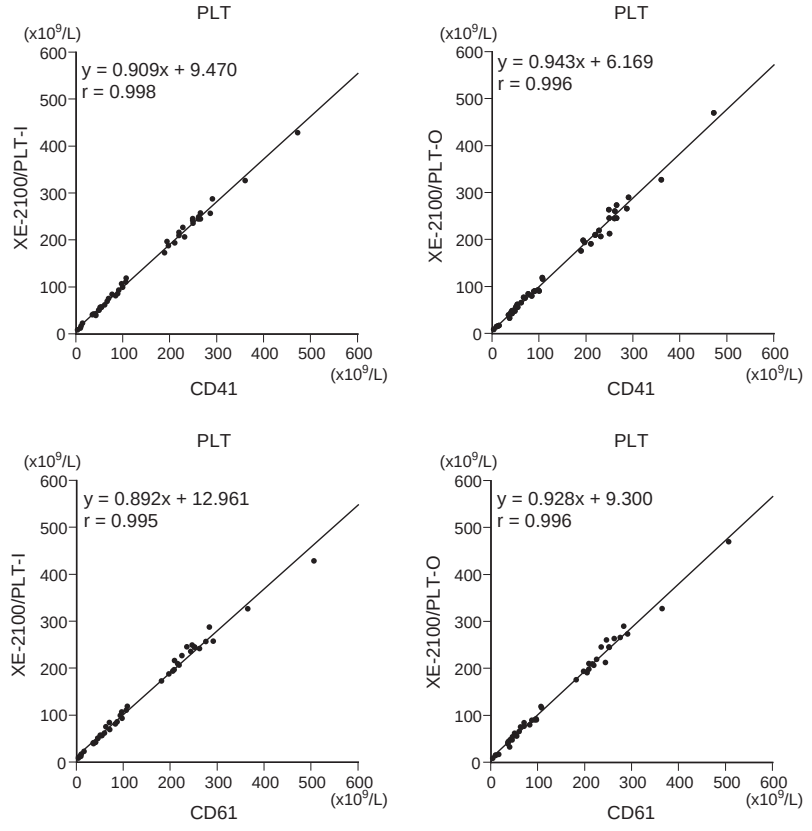


Fig. 4 *Correlation with FCM*

DISCUSSION

Using samples from normal subjects and thrombocytopenia samples, a comparison was done between FCM employing monoclonal antibody (reference method for PLT enumeration) and PLT data obtained from the XE-2100. This comparison showed a very good correlation between the two. From the results of our present study, any differences were not seen in the data regardless of the PLT enumeration method that was used. Examples of PLT enumeration by each method are shown in ***Fig. 5***. Hereafter, it will be necessary to investigate samples with abnormal cells such as large PLT and schistocytes.

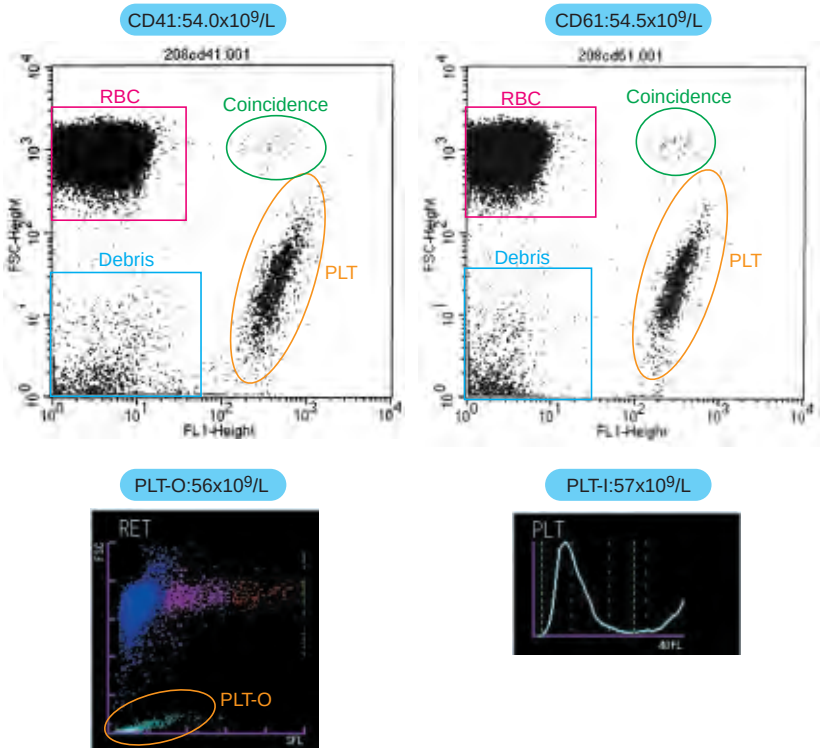


Fig. 5 *Examples of platelet enumeration data*

2nd report: Report of enumeration data in abnormal samples (samples with large platelets etc.)

INTRODUCTION

In the 1st report, we reported on our enumeration of PLT by a monoclonal antibody-employed method proposed by the ICSH, and on comparison of its results with the PLT-I count obtained from the XE-2100, an automated Hematology analyzer employing electric resistance system, and another PLT-O count obtained by optical system (See the 1st report). In this 2nd report, we report on our study of samples that may affect the size distribution of PLT. This method has recently been reported as ICSH's reference method[7].

SAMPLES

K_2EDTA samples from patients: n=28 (tube used for blood collection: Venoject II, Terumo).
In this study, we used samples that might affect size distribution of PLT, such as samples with large PLT, nucleated red blood cell (NRBC), schistocytes, and those samples with suspected disseminated intravascular coagulation (DIC). However, samples with a large aggregation of PLT were excluded from the study, since it is impossible to make an accurate enumeration of PLT by Fonio method in these samples. Enumeration was made within 24 hours after blood collection, and samples were kept at room temperature until the time of enumeration.

ENUMERATING INSTRUMENT

FACSCalibur (Becton Dickinson), the XE-2100 (Sysmex Corporation, a standard counter at Scientific Division).

REAGENTS USED

Monoclonal antibody: CD41-FITC (Beckman Coulter); CD61-FITC (Becton Dickinson); Simultest Control γ_1/γ_2a (negative control, Becton Dickinson).
Diluent: PBS containing 0.1% BSA.

DETAILS OF ENUMERATING METHOD

See the 1st Report.

RESULTS

Correlation between Fonio method and each enumerating method

Correlation with Fonio method was studied in samples showing abnormality in size distribution of PLT. Using CD41 in FCM, the regression equation was y=1.169x–1.304 with a correlation coefficient of r=0.995; and where CD61 was used, y=1.048x + 7.747 in regression equation, with a correlation coefficient of r=0.986. Similarly, when the XE-2100 was used, comparison with PLT-I data was a regression equation of y=0.991x + 0.290 with a correlation coefficient of r=0.997. With PLT-O data, the regression equation was y=0.947x + 6.530 with a correlation coefficient of r=0.995 (***Fig. 6***). Since the correlation coefficient showed 0.98 or greater, this was a very good correlation.

Correlation between FCM and XE-2100

Correlation between FCM and the XE-2100 was studied in samples showing abnormal size distribution of PLT. When CD41 and PLT-I were used, the regression equation was y=1.172x–0.598 with a correlation coefficient of r=0.992. With PLT-O, the regression equation was y=1.217x–5.696 with a correlation coefficient of r=0.985. Similarly, where CD61 and PLT-I were used, the regression equation was y=1.047x + 9.183 with a correlation coefficient of r=0.979. With PLT-O, the regression equation was y=1.086x + 4.519 with a correlation coefficient of r=0.972 (***Fig. 7***). Thus, the correlation coefficient was 0.97 or greater in all cases, indicating a very satisfactory correlation.

Correlation between PLT-I and PLT-O in XE-2100

Correlation between PLT-I and PLT-O in the XE-2100 was studied in samples showing abnormal size distribution of PLT. As a result, the regression equation was y=1.041x–5.640 with a correlation coefficient of r=0.996, indicating very good correlation (***Fig. 8***).

DISCUSSION

Using samples that show abnormality in size distribution of PLT, a comparison was made between PLT data by monoclonal antibody-employed FCM (reference method for PLT enumeration) and PLT data by the XE-2100. As a result, very good correlation was observed. It is said that data reliability is low in PLT enumeration by electric resistance system if samples used are such that they may bring about abnormality in size distribution of PLT. However, from the results of our present study, no differences were observed in obtained data regardless of sample, no matter which enumerating method was used.
In regards to the PLT enumeration by electric resistance system, there was considerable improvement made in the algorithm for PLT upper discriminator.
As an example of enumeration in a sample showing giant PLT (***Picture 1***), ***Fig. 9*** is shown for reference. It shows enumeration data of each method in samples showing giant PLT. However, it should be noted that we have not been able to examine all cases of abnormal size distribu-

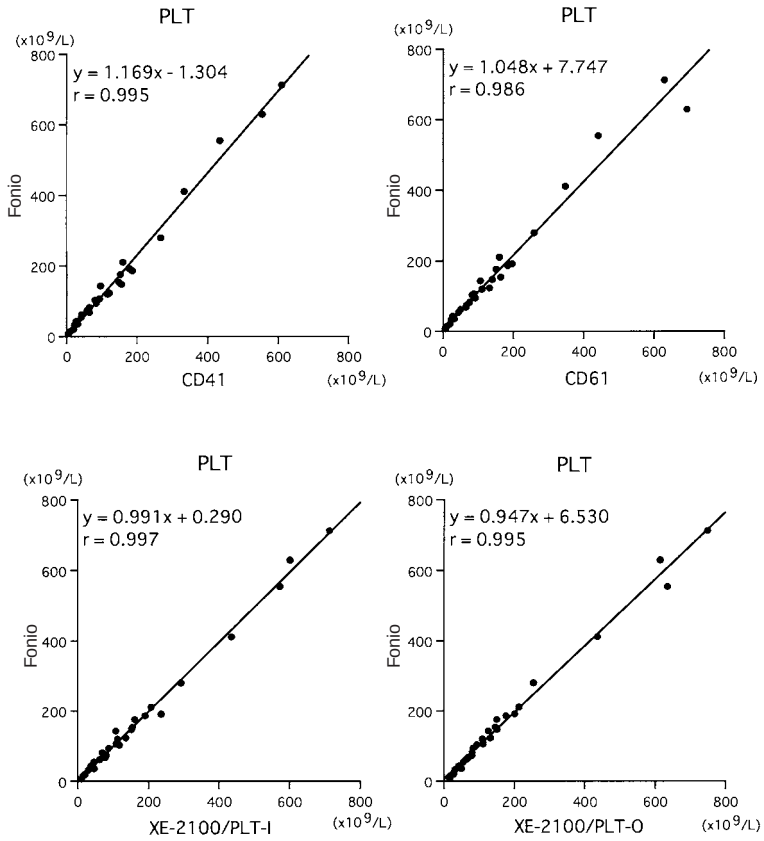


Fig. 6 *Correlation with Fanio method*

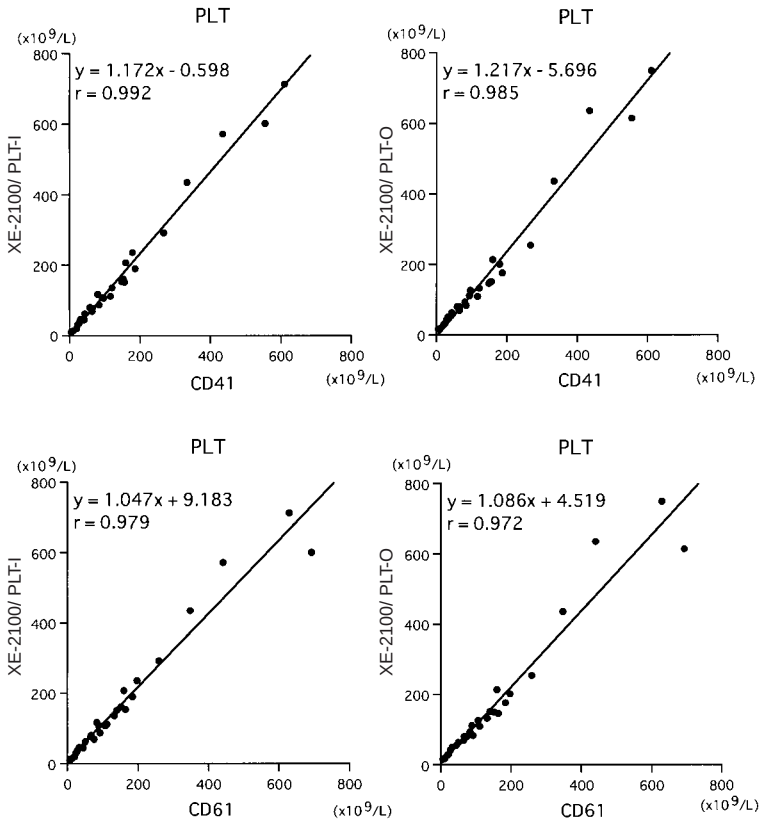
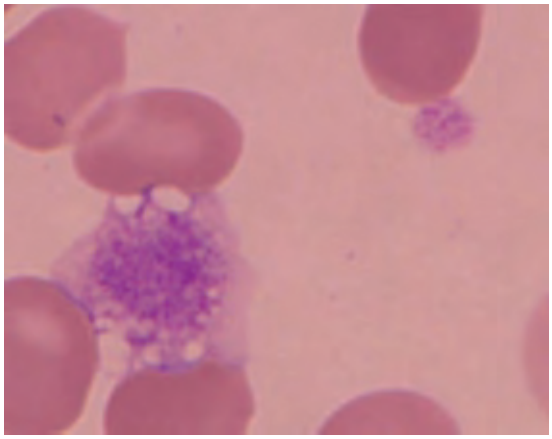


Fig. 7 *Correlation with FCM*



Picture 1 *Giant PLT*

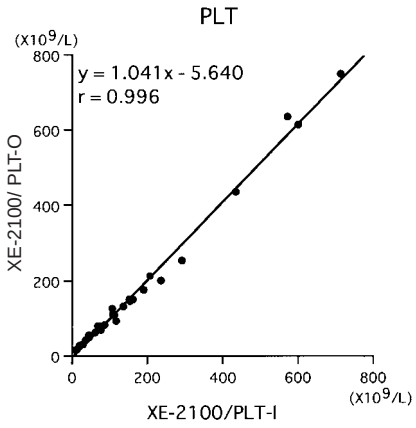


Fig. 8 *Correlation between PLT-I and PLT-O*

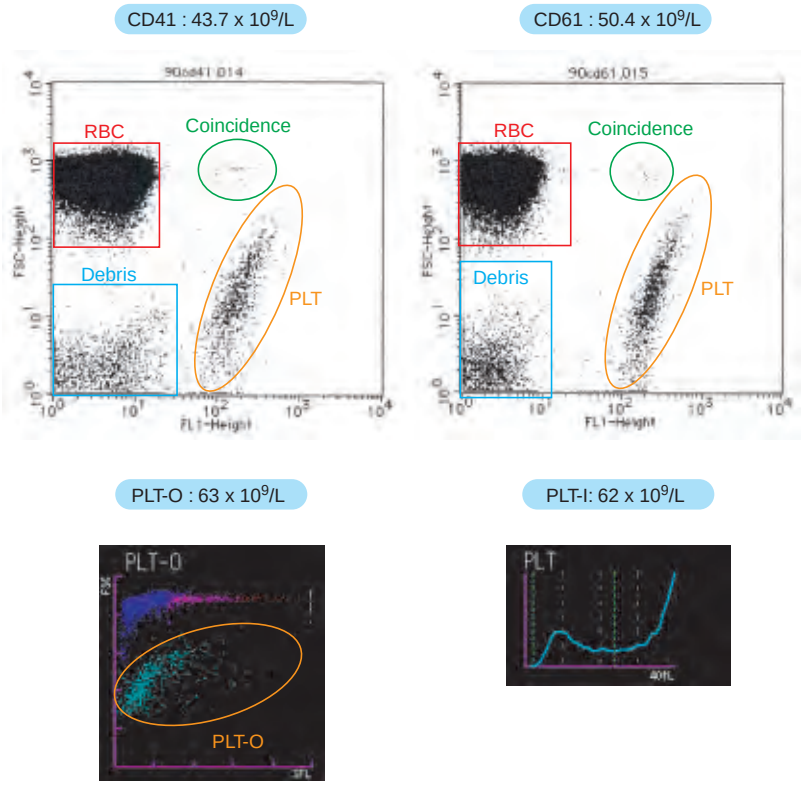


Fig. 9 *Example of enumeration in a sample showing Giant PLT*

tion of PLT. Thus, for samples with remarkable abnormal size distribution of PLT, further investigation is needed.

If, in the future, we have a chance of studying samples in which data difference is observed between PLT-I and PLT-O, we will report the results.

Reference

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