Analisador automático de células com citometria de fluxo – 
Precisão na contagem de plaquetas e triagem de doenças 
linfoproliferativas crônicas B

Marciano Reis, MD, FRCPC
Chief, Dept. of Clinical Pathology
Sunnybrook Health Sciences Centre
Women’s College Hospital
Chief, Dept. of Laboratory Hematology
University Health Network
Associate Professor
University of Toronto

HIAE, April 2009
Platelet count: Accuracy by hematology analyzers with flow cytometry capabilities

Abbott CELL-DYN
- Sapphire
- 4000
- solid-state blue diode laser at 488 mm
- dual angle optical analysis
- counts validated by impedance
- CD61: random access; automated

Sysmex HE-2100
HE-5000

Sysmex – Polymethine and oxazine florescent dye to stain RNA/DNa, of reticulated cells and platelet membrand and granules.
- allows simultaneous counting of reticulotyes, erythrocytes and platelets
• Works in an identical way to Standard Flow Cytometers

• An Argon Ion laser (10mW output with a 488nm wavelength) is focused onto the stream of cells and the resultant emission wavelengths are monitored after passage through further filters corresponding to FL1, FL2, and FL3

• Combined with Multi Angle Polarized Scatter Separation - MAPSS
Analytical Approach
Challenges In Platelet Counting

False Increases
- RBC fragments
- Microcytes
- Bacteria
- Immune complexes
- Leukocyte fragments
- Chylomicrons

False Decreases
- Giant platelets
- Platelet clumps

Dual Angle Optical Platelet Analysis
Impedance Count Limitations

- Analyzers using standard impedance measurements are able (for most samples) to provide an accurate platelet count down to $20 \times 10^9$/L.
- Below this level, impedance analyzers become increasingly inaccurate because of:
  - Loss of linearity
  - Decreasing statistical confidence (i.e. fewer events actually measured)
  - Increasing influence of background and plasma non-platelet particulate matter

Immuno-Platelet Analysis
Why do clinicians need another platelet count method?

- "Clinicians who utilize platelet thresholds of 10 or 5 x 10^9/L must be aware of the limitations in precision and accuracy of cell counters at this level."
- "Accurate counting of low platelet numbers may create difficulties when trying to reduce the threshold below 10 x10^9/L"

Consensus statement on Platelet Transfusion Therapy.
Royal College of Physicians Consensus Conference
Norfolk D.R et al
Brit J Haem 1998,101,609-617

Immuno-Platelet Analysis
Reference Method for Platelet Enumeration


- Main principle for analyzing platelets by automated hematology analyzer → electric resistance (impedance)

- Tanaka & Fujimoto: studied platelets with normal size distribution, comparing FCM, impedance and optical (PLT-O) counting by the Sysmex HE-2100
  - Normal counts and thrombocytopenic samples

Figure 4

Fig. 4  Correlation with FCM
Enumeration of platelets in samples with abnormal (large) platelets

Fig. 7

Correlation with FCM
Letter to the Editor: The most accurate platelet count on the Sysmex XE-2100. Optical or impedance?

- Impedance count always provided; optical fluorescence count if run in reticulocyte mode

- A switching algorithm mode to report the most “correct” platelet count (optical or impedance)

- Samples with microcytes or fragmented RBCs → the platelet distribution curve is affected and the optical count will be reported

- Sample with WBC fragments (included in the optical platelet count) → abnormal scatter gram flag → switching algorithm causes the impedance count to be reported
Briggs et al: The most accurate platelet count at low levels (<20 x 10^9/l), on the XE-2100, is not always the optical count

- Common practice to assume that the optical count is more accurate in all samples with low counts

- Widespread overriding of the switching algorithm and setting the analyzer to report the optical count at preset level (typically 50 x 10^9/l).

- This should be discouraged: The XE-2100 gives the most accurate platelet count when the switching algorithm is allowed to report the most accurate count

- Large platelets → excluded from the impedance count on the basis of size
Study of 501 chemotherapy samples (platelets < 20 x 10^9/l); optical, impedance and FCM method

- 232 samples → no platelet flags (normal impedance platelet distribution curve)
  - in all → switching algorithm chose the impedance count
    - impedance vs FCM \( r^2 = 0.80 \)
    - optical vs FCM \( r^2 = 0.72 \), likely because of WBC fragments
269 samples with platelet flags

- 105 (39%): optical counts reported, with $r^2 = 0.84$
- impedance counts: $r^2 = 0.73$
  thus, the optical count was more accurate for those samples

- 164 (61%): impedance counts reported, with $r^2 = 0.79$
  if optical count reported: $r^2 = 0.72$, with some high false counts

- Conclusion: The accuracy of the XE-2100 platelet count is excellent for low platelet counts if the switching algorithm is used
IPF as a guide to platelet transfusion requirement after hematopoietic stem cell transplantation:


- Established normal range in 50 healthy subjects
- IPF measured in pts receiving chemotherapy (30) or post autologous or allogeneic HSC transplant (15)
- prophylactic platelet transfusions if counts fall to 5-20 x 10^9/l, in the absence of fever; at higher levels for invasive procedure
- some groups now considering therapeutic rather than prophylactic txs
- ability to predict reliably when platelet recovery will occur → should allow a more reasoned approach to prophylatic tx
- reticulated platelets: larger, more physiologically active; analogous to reticulocytes; their proportion reflects the rate of thrombopoiesis
- ↑ in IPF% was the first indication of platelet recovery
- rise is earlier in pts undergoing PBSCT vs bone marrow-derived HSC
Immature Platelet Fraction

IPF% - proprietary and FDA-licensed name of high fluorescence platelet fraction percentage (HFPF%) measurement by Sysmex XE-2100
- software to discriminate two platelet populations:
  mature (blue dots) and immature (red dots) platelets

Fig 1
Evaluation of HFPF% in Thrombocytopenia

- Used the XE-2100 to quantify HFPF% as an indicator of platelet production
- 80 healthy subjects with normal platelet count:
  - HFPF% 3.1 (95% CI 2.8%-3.5%)
  - CV 10-20%
- Sample stability up to 48 hours
- 171 thrombocytopenic patients
  - Highest values in DIC and autoimmune thrombocytopenia; also increased in pts. with regenerating bone marrows
  - Within the normal range in pts. with decreased platelet production
## Table 2
High Fluorescent Platelet Fraction Percentage

<table>
<thead>
<tr>
<th>Subject</th>
<th>No. of Cases</th>
<th>Mean</th>
<th>SD</th>
<th>Range</th>
<th>95% Confidence Interval</th>
<th>P*</th>
</tr>
</thead>
<tbody>
<tr>
<td>Healthy Destruction</td>
<td>80</td>
<td>3.1</td>
<td>1.6</td>
<td>1.0-7.0</td>
<td>2.8-3.5</td>
<td>—</td>
</tr>
<tr>
<td>ATP</td>
<td>37</td>
<td>15.0</td>
<td>7.1</td>
<td>3.6-35.4</td>
<td>12.7-17.2</td>
<td>&lt;.0001</td>
</tr>
<tr>
<td>DIC</td>
<td>25</td>
<td>9.5</td>
<td>5.2</td>
<td>2.3-18.6</td>
<td>7.5-11.5</td>
<td>&lt;.0001</td>
</tr>
<tr>
<td>All destruction</td>
<td>62</td>
<td>12.8</td>
<td>6.9</td>
<td>2.3-35.4</td>
<td>11.0-14.5</td>
<td>&lt;.0001</td>
</tr>
<tr>
<td>Recovery</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Chemotherapy</td>
<td>79</td>
<td>4.1</td>
<td>2.0</td>
<td>1.2-10.9</td>
<td>3.6-4.5</td>
<td>.001</td>
</tr>
<tr>
<td>BMT</td>
<td>8</td>
<td>8.0</td>
<td>4.2</td>
<td>4.3-15.3</td>
<td>5.1-10.8</td>
<td>&lt;.0001</td>
</tr>
<tr>
<td>All recovery</td>
<td>87</td>
<td>4.4</td>
<td>2.5</td>
<td>1.2-15.3</td>
<td>3.9-4.9</td>
<td>&lt;.0001</td>
</tr>
<tr>
<td>Suppression</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>AA/PNH</td>
<td>3</td>
<td>6.1</td>
<td>2.5</td>
<td>3.9-8.8</td>
<td>3.2-8.9</td>
<td>.019</td>
</tr>
<tr>
<td>Cancer</td>
<td>16</td>
<td>3.8</td>
<td>2.0</td>
<td>0.8-7.1</td>
<td>2.8-4.7</td>
<td>NS</td>
</tr>
<tr>
<td>All suppression</td>
<td>19</td>
<td>4.1</td>
<td>2.2</td>
<td>0.8-8.8</td>
<td>3.1-5.1</td>
<td>.05</td>
</tr>
</tbody>
</table>

ATP: autoimmune thrombocytopenia; AA/PNH: aplastic anemia/paroxysmal nocturnal hemoglobinuria; BMT: bone marrow transplantation; DIC: disseminated intravascular coagulation; NS: not significant.

* P values are vs healthy. Mann-Whitney U test, 2-sided. In addition to the P values given, the high fluorescent platelet fraction percentage was significantly higher (P < .0001) in patients with platelet destruction (ie, ATP or DIC) than in thrombocytopenic patients with recovery marrows or in those with suppressed marrows (P < .0001). Patients with ATP had higher high fluorescent platelet fraction percentage values than did patients with DIC (P = .002).
Thus, HFPF% predictive in the evaluation of thrombocytopenia:
- elevated in association with increased production
and normal with decreased platelet production
ImmunoPlt™ (CD61) Assay

- Reagent
  - Proprietary lyophilized formulation
  - Unique matrix allows rehydration without aggregate formation.
  - Excellent room temperature stability.
  - Unit Test Packaging so NO WASTE

Immuno-Platelet Analysis

CELL-DYN®
The main conclusion was that CD61 ImmunoPLT method was more accurate than any other technology. This finding agreed with other studies comparing the CD61 ImmunoPLT method with the FCM reference method.
### Table 4

Reproducibility of Analysers and Immunocounting in Severe Thrombocytopenia

<table>
<thead>
<tr>
<th>Analyser type</th>
<th>CV (%) at 5 x 10⁹/L</th>
<th>CV (%) at 10 x 10⁹/L</th>
</tr>
</thead>
<tbody>
<tr>
<td>Flow cytometry</td>
<td></td>
<td></td>
</tr>
<tr>
<td>RBC/platelet ratio</td>
<td>3.83</td>
<td>5.65</td>
</tr>
<tr>
<td>XE2100</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Optical Impedance</td>
<td>6.62</td>
<td>8.02</td>
</tr>
<tr>
<td></td>
<td>20.14</td>
<td>10.05</td>
</tr>
<tr>
<td>Cell Dyn 4000</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Optical ImmunoPLT CD61</td>
<td>12.3</td>
<td>3.8</td>
</tr>
<tr>
<td></td>
<td>1.6</td>
<td>2.3</td>
</tr>
<tr>
<td>Advia 120</td>
<td>12.5</td>
<td>13.2</td>
</tr>
<tr>
<td>LH 750</td>
<td>20.26</td>
<td>8.78</td>
</tr>
</tbody>
</table>

Samples were analysed 10 times by each test and the CV was calculated from the formula (standard deviation/mean) x 100. Unpublished data were derived from Segal et al. (12).
Reducing Interferences In The Platelet Count

CD61 Counting

<table>
<thead>
<tr>
<th>WBC</th>
<th>72.7 10⁶/L</th>
</tr>
</thead>
<tbody>
<tr>
<td>NEU</td>
<td>10.7%</td>
</tr>
<tr>
<td>LYM</td>
<td>50.6%</td>
</tr>
<tr>
<td>MONO</td>
<td>11.0%</td>
</tr>
<tr>
<td>EOS</td>
<td>.185%</td>
</tr>
<tr>
<td>BASO</td>
<td>.148%</td>
</tr>
</tbody>
</table>

CD61 Co |

.87

IG .87

VARLYM .60

RBC 2.81 s/dl

HGB 8.68 g/dl

HCT 289 s/dl

MCV 103 s/dl

MCH 30.9 s/dl

MCHC 30.0 s/dl

RDW 26.0 s/dl

RETIC .04% s/dl

IRF .01

NRBC 1.05 10⁶/L

PLT 7.12 10⁹/L

MPV 12.7 s/dl

PDW 31.6 s/dl

PCT .090% s/dl

PLT 40.3

CD61 1.843

PLTs 0.00

PLTl 0.00

Optical PLT

Impedance PLT

Immuno-Platelet Analysis/WBC Fragmentation
CD61/41 analysis in a family with Glanzmann’s Thrombasthenia.
CELL-DYN Sapphire open mAb assay

Peak spectral fluorescence of the CELL-DYN Sapphire achieved with the blue (488nm) diode-pumped solid-state laser.
Automated CD 3/4/8 mAb Assay

• Fully Automated
• Fully Enclosed

Step 1

Step 2

Automated CD 3/4/8 Analysis
Automated CD 3/4/8 mAb Enumeration
**CD-Sapphire Extended Applications**

<table>
<thead>
<tr>
<th>User-Defined Assay:</th>
<th>Empty</th>
<th>100uL EDTA Blood Mab-FITC Mab-PE</th>
<th>100uL EDTA Blood Mab-FITC Mab-PE</th>
</tr>
</thead>
</table>

**WBC Antigens**
CD-Sapphire Extended Applications

- FCS Express v3 flow cytometry software has been designed to directly open CD-Sapphire fcs files.

- For the qualitative analysis of lineage-associated antigens, the gating strategy is straightforward and intuitive.
MAb titration

3/4, monocytes

T and NK (2+, 3-)

16/56 NK fractions

DR, 11b, 13, 33  lymphs, granulocytes, monocytes

• For most samples, the vast majority of lymphocytes were shown to be T-or B-cell in nature. However, for some samples there was clear evidence for an additional significant non-T, non-B components.
Passing and Bablok agreement analysis
• **Case Example 1**

Sample with lymphocyte count of 10.1 with otherwise normal haematological picture.
Extended Monoclonal Antibody Applications

CD3+ T-cells: $2.7 \times 10^9$/L  
CD19+ B-cells: $6.0 \times 10^9$/L
Case Example 2
Sample with lymphocyte count of 8.9 with otherwise normal haematological picture.
Extended Monoclonal Antibody Applications

WBC Differential

<table>
<thead>
<tr>
<th>X-B</th>
<th>WBC</th>
<th>RBC</th>
<th>PLT</th>
<th>RETC</th>
</tr>
</thead>
<tbody>
<tr>
<td>In</td>
<td>In</td>
<td>In</td>
<td>In</td>
<td>In</td>
</tr>
<tr>
<td>WBC</td>
<td>13.5</td>
<td>10c3/uL</td>
<td>WVF</td>
<td>.980</td>
</tr>
<tr>
<td>NEU</td>
<td>2.75</td>
<td>%N</td>
<td>20.4</td>
<td></td>
</tr>
<tr>
<td>LYM</td>
<td>8.88s</td>
<td>%L</td>
<td>65.9s</td>
<td></td>
</tr>
<tr>
<td>MONO</td>
<td>1.66s</td>
<td>%M</td>
<td>12.3s</td>
<td></td>
</tr>
<tr>
<td>EOS</td>
<td>.117</td>
<td>%E</td>
<td>.866</td>
<td></td>
</tr>
<tr>
<td>BASO</td>
<td>.070</td>
<td>%B</td>
<td>.523</td>
<td></td>
</tr>
</tbody>
</table>

BLAST 0.77
VARLYM 0.70

CD3

CD 19

CD3+ T-cells: $8.5 \times 10^9/L$
CD19+ B-cells: $0.1 \times 10^9/L$
Extended Monoclonal Antibody Applications

Case Example 3
Sample with lymphocyte count of 6.3, normocytic anaemia and mild thrombocytosis.
The immunological profile of lymphocytes in this sample show:

A marked increase in the proportion of CD2+CD3- lymphocytes

Normal relative proportions of T-Helper to T-Suppressor cells, and T-cells to B-cells.

Confirmation (CD16/CD56) that the abnormal component is NK in origin
CELL DYN vs. FCM

- **advantages**
  - FCM lab can concentrate on leukemias/lymphomas
  - no need for FCM training
  - 24 / 7

- **disadvantages**
  - no EQA materials yet
  - Costs: $3 Cdn. dollars more per CD3/4/8 tests
Substituting red cell anti-Glycophorin A (CD235A) in the CD-Sapphire automated immunoplatelet assay allows the qualitative demonstration of red cell schistocytes.
Figure II. Analysis of platelet glycoprotein expression. Upper plots (a) to (e) show MFI vs. 7A optical scatter profiles for normal platelets stained with antibodies against platelet glycoprotein Ibα (CD41), Ibβ (CD42b), and IIIa (CD61). The green events are antigen-positive platelets, with the green events within the red ellipse corresponding to red cell/platelet coincident events. The lower plots show the colour-coded platelet counts according to their two-dimensional 90° vs. 7A optical scatter profiles. Using the same dye procedures, an antibody against red cell glycophorin A (CD235a) can alternatively be used for the detection of red cell fragments (schistocytes) in the platelet region of the two-dimensional optical plot. In this example (d), the platelet population is seen as an elliptical population of black events while the coexisting red cell fraction (upper right region) is CD235a+ and colour-coded red. Procedures started out using 100 μl of EDTA-citrate anticoagulated blood mixed with 5 μl of monoclonal antibody reagents; flow cytometric analysis using WINMDI.