AUTOMATED RETICULOCYTE ANALYSIS

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Reticulocyte

- The last immature erythrocyte stage
- Spend 2-3 days in marrow and 1 day in circulation $\rightarrow$ mature erythrocyte
- Contain remnant cytoplasmic RNA and organelles (mitochondria & ribosomes)
- Retics = non-nucleated erythrocyte that contains $\geq 2$ particles of blue-stained granulofilamentous material after NMB-staining
Fig. 1. Reticulocyte (New Methylene Blue staining)
The amount of reticulocytes in circulation reflects erythropoietic activity.

Retic. maturation started from extrusion of orthochromatic normoblast nucleus, to complete loss of ribosomes and RNA, is thought to take 4 days (only the last day occurs in the circulation).
Fig. 2. Erythropoiesis stages
1930: **Heilmeyer** classify 4 groups of reticulocytes:

- **Grup 0** - normoblast
- **Grup I** - Reticulum as a clumped precipitate (0.1%)
- **Grup II** - Reticulum as a form of wreath (7%)
- **Grup III** - show an opened wreath (32%)
- **Grup IV** - only shown a few granules of the reticulum (61%)
Fig. 3. Stages of Red Cell Maturation
Fig. 4. Heilmeyer’s reticulocytes maturation stages
Retic.count Reporting Methods

1. **Percentage**:
   % Retics/ 1000 red cells (N: 1±0.5%)

2. **Corrected Retics**
   - correction made for anemic patients
   - increased retic.count:
     → increased of retics in circulation
     → decreased of red cells in circulation
   - observed retic.count corrected to normal Hct / PCV (0.45)
Corrected Retic.count = \frac{\text{patient's PCV} \times \text{observed retics(\%)} \times 0.45}{\text{Corrected Retic.count}}
3. **Absolute Retic.Count**

4. **Retic.Production/Maturation Index:**
   - peripheral retic.number is combination of the *rate of release* of retics from marrow and the *degree of immaturity* of freshly released retics.
In ↑ erythrop’s stimulation:

1. younger retics (shift cells) released into circulation ( = basophilic macroretics)

2. shortened retic. maturation time in marrow and longer maturation time in circulation
Fig. 5. Reticulocyte’s Maturation time

- PCV
- Bone Marrow
- Peripheral Blood
- .45
  - 3.5
  - 1.0
- .35
  - 3.0
  - 1.5
- .25
  - 2.5
  - 2.0
- .15
  - 1.5
  - 2.5
Reticulation Production/Maturation Index = 

\[
\text{Pt's PCV} \times \frac{\text{observed retic.count} \%}{0.45} \times \text{maturation time in circulation}
\]
Younger Retics (Shift Cells)

- Reticulocyte = red cell containing ≥ 2 stained intra erythrocytic particles
- Circ.Retics are not distinguishable from mature red cells morphologically in Wright-stained smears
- Young retics (shift cells) have the greatest quantity of RNA
Young retics (Shift cells)

- Young retics have a bluish cast after fixation and staining with Wright’’ stain, larger than mature red cells, called Polychromatophilic macrocytes (N: < 5% of total Retics)
- Polychr.macrococytes = a good indication of EPO-mediated increase in erythropoiesis
Young Retics (Shift Cells)

- Hb level of **10.5 g/dl** (Hct 31%) is the critical threshold for increased Polychromatophilic macrocytes
Assessment of Manual Retic.Count:

- High interlab’s CV (25-48%) due to:

  1. interobserver variation in retic’s definition
  2. the number of red cells evaluated
  3. the types of blood film stained
  4. the use of standard area-reduction device (Miller disc)
### Table 1. The number of counted erythrocytes and reticulocytes’ precision

<table>
<thead>
<tr>
<th>Retic.count(%)</th>
<th>CV 2%</th>
<th>CV 5%</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>27,700</td>
<td>4,500</td>
</tr>
<tr>
<td>3</td>
<td>11,100</td>
<td>1,350</td>
</tr>
<tr>
<td>5</td>
<td>7,750</td>
<td>1,100</td>
</tr>
<tr>
<td>10</td>
<td>5,000</td>
<td>900</td>
</tr>
<tr>
<td>20</td>
<td>4,000</td>
<td>650</td>
</tr>
<tr>
<td>30</td>
<td>3,500</td>
<td>550</td>
</tr>
<tr>
<td>40</td>
<td>3,000</td>
<td>500</td>
</tr>
</tbody>
</table>
- Manual reticulocytes count:
  - imprecise
  - inaccurate
  - labour intensive →

  cannot yield a quantitative measurement.
Clinical interests

↓

only on ↑ retics
(in hemorrhage, hemolysis, hematinic therapy’s response)

↓

imprecision/inaccuracy was tolerable
- **Modern medicine** →
  increase of reticulocyte’s clinical utilities → need **more precise & accurate** retic. counts
Reticulocyte’s Clinical Utilities

1. For hematological diagnosis:

- Classify anemic patients
- Assess bone marrow’s function
- Aplastic crisis
- Myelodysplastic Syndrome (MDS)
- Hemorrhages or hemolysis
2. Treatment Monitoring:

- In EPO therapy
- As an indicator of Marrow’s regeneration after chemotherapy or BMT
- Timing the Stem Cell harvest
Automated reticulocytes count is more accurate
(Manual : CV $> 25\% \rightarrow 5\%-7\%$)

Blood cell counters permit precise measurements of RNA content and cellular indices (Volume, Hb-concentration and Hb-content)
CV from manually Reticulocyte count & Analyzers:

<table>
<thead>
<tr>
<th></th>
<th>CV Manual</th>
<th>CV Automatic</th>
</tr>
</thead>
<tbody>
<tr>
<td>Reticulocyte 1%</td>
<td>47.3%</td>
<td>6.4%</td>
</tr>
<tr>
<td>Reticulocyte 9%</td>
<td>27.2%</td>
<td>5.8%</td>
</tr>
</tbody>
</table>
Hematology’s Automation

- 2 basic principles operation of Hematology Analyzers:

1. Electronic Impedance / low-voltage DC resistance (Coulter, 1950s)

2. Optical Scatter
Reticulocyte’s Automation

- Earlier there were only 2 automatic methods for counting reticulocyte:
  - Computer-controlled automated microscope for blood smear analysis (early ’80s)
  - Flow cytometric methods
Automatic microscope → analogue with manual light-microscope, and scanned automatically NMB-stained blood films using a pattern recognition devices.

→ a good equipment for its better reproducibility, the analysis results is as good as the manual methods. Unfortunately it is not so popular.
Automatic retic. Count method using acridine-orange fluorescence (1952) → the dye binds to retic’s ribosomal-RNA → fluorescent in UV-light.

→ the intensity of fluorescence is proportional to the RNA present → ~ maturity of the reticulocytes.

There is satisfactory agreement between manual and automated methods in categorizing the 4 Heilmeyer maturation groups.
- Fluorescence staining combined with flow-cytometry → led to the new automated systems for reticulocyte counting.

- Several different dyes have been used for flow cytometric retics count.
Flow cytometric Retic. counting

- Many fluorochromes have been used, i.e.:
  - Acridine orange
  - Auramine O
  - Di-methylloxacarbocyanide
  - Ethidium bromide
  - Pyronin - Y
  - Thioflavine-T
  - Thiazole orange
most require 30 minutes incubation → semi-automated?
- **Ethidium bromide** at relatively high pH require only few minutes to enter the cells

- **Auramine O** requires only a few seconds
Cell Information - FCM

- Side scatter: Information on internal structure
- Fluorescence: Information on amount of RNA and DNA
- Forward scatter: Information on cell size

Laser Beam
### Table 2. Evolution on parameters’ content of CBC

<table>
<thead>
<tr>
<th>No</th>
<th>Parameters</th>
<th>Year</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Hb, Hct, RBC, WBC</td>
<td>1950s</td>
</tr>
<tr>
<td>2</td>
<td>MCV, MCH, MCHC</td>
<td></td>
</tr>
<tr>
<td>3</td>
<td>PLT, RDW, PDW, MPV, Pct, P-LCR, RDW-SD</td>
<td></td>
</tr>
<tr>
<td>4</td>
<td>LUC, CHCM, HDW, LI, MPXI</td>
<td>1960s</td>
</tr>
<tr>
<td>5</td>
<td>LYM, MONO, GRAN (%, #)</td>
<td>1970s</td>
</tr>
<tr>
<td>6</td>
<td>NEUT, EOS, BASO (%, #)</td>
<td>1980s</td>
</tr>
<tr>
<td>7</td>
<td>FLAGGING</td>
<td></td>
</tr>
<tr>
<td>8</td>
<td>RETICULOCYTE COUNT</td>
<td>1990s</td>
</tr>
</tbody>
</table>
Current Options on Reticulocyte Counting:

<table>
<thead>
<tr>
<th>Method</th>
<th>Dye</th>
<th>Technique</th>
<th>Usage</th>
<th>CV-%</th>
</tr>
</thead>
<tbody>
<tr>
<td>Microscopy, Supravital</td>
<td>NMB/BCB</td>
<td>Manual</td>
<td>± 70%</td>
<td>25</td>
</tr>
<tr>
<td>Flow Cytometry</td>
<td>Thiazole O</td>
<td>Fluorescence</td>
<td>&lt; 1%</td>
<td>15-20</td>
</tr>
<tr>
<td>Flow Cytometry</td>
<td>NMB, spered</td>
<td>Optical scatter</td>
<td>± 10%</td>
<td>5-10</td>
</tr>
<tr>
<td>Flow Cytometry</td>
<td>Oxazine 750</td>
<td>Optical scatter</td>
<td>± 4%</td>
<td>5-10</td>
</tr>
<tr>
<td>Flow Cytometry</td>
<td>Auramine O</td>
<td>Fluorescence</td>
<td>± 5%</td>
<td>5-10</td>
</tr>
</tbody>
</table>
# Retic.count methods on Hematology instruments

<table>
<thead>
<tr>
<th></th>
<th>Coulter LH-750</th>
<th>Sysmex XE-2100</th>
<th>Abbott CD-4000</th>
<th>Bayer Advia 2120</th>
</tr>
</thead>
<tbody>
<tr>
<td>Supravital staining (NMB)</td>
<td>Supravital staining (Auramin O) Fluorescent detection</td>
<td>CD4K530 stain Multiangle scatter and Fluorescent detection</td>
<td>Supravital staining (Oxazine 750) Low-angle (2-30) and High-angle (5-150, optical scatter and absorbance)</td>
<td></td>
</tr>
</tbody>
</table>
The improved precision for Flow Cytometric methods arises from:

1. Removal of inter-observer variation

2. The larger number of cells counted (10000 – 30000 RBC events, compare to 1000 for the visual method)

3. The fluorescence measured is proportional to the amount of RNA present in the cell
How the methods inform the maturation of Retics?

- The analyzers divide the retic area on the scattergram by 2 vertical discriminators → producing 3 populations:
  - Low Fluorescence Ratio (LFR) – the most mature forms
  - Middle Fluorescence Ratio (MFR)
  - High Fluorescence Ratio (HFR) – the least mature forms
Fig. 6. Frequency’s curve of red cell maturation

RMI = Mean fluorescence of Retics

IRF = MFR + HFR / (LFR + MFR + HFR)
Reticulocyte’s Automation

- **Fluorescence-based** (Thiazole Orange, Auramine O, fluorescence’ dyes)
- **Absorbance-based** (Oxazine O, New Methylene Blue, nucleic acid dyes)
- Interacting with RNA
- Immature Retics → high fluorescence
- **Thiazole Orange** → overestimated because of DNA / RNA content of another cells/components
### Table 3. Reticulocyte’s parameters in various hematology analyzers

<table>
<thead>
<tr>
<th>Methods</th>
<th>Parameters</th>
</tr>
</thead>
<tbody>
<tr>
<td>CD 4000 (Abbott)</td>
<td>Fluorescen (Auramine O)</td>
</tr>
<tr>
<td></td>
<td>Absorbans (New Methylene Blue)</td>
</tr>
<tr>
<td>SE-9000 (Sysmex)</td>
<td>Fluorescen (Auramine O)</td>
</tr>
<tr>
<td></td>
<td>Absorbans (Oxazine 750)</td>
</tr>
</tbody>
</table>
Fig. 7. Reticulocyte Analysis

670nm Laser Diode

Oxazine 750 RNA Stain

High angle detector (5° - 15°)

Low angle detector (2°-3°)

Absorbance RNA Content
Fig. 8. Reticulocyte Technology

- Reticulocytes are stained with a nucleic acid dye - Oxazine 750
- Scatter and Absorption are measured using laser channel
- RBC and Reticulocyte indices are measured simultaneously
Immature Reticulocytes = MFR + HFR

Assist in earlier diagnosis of anemia
Fig. 9. Reticulocyte Cytogram and Relationship to Maturity

Oxazine 750 Absorbance
Fig. 10. CBC results

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Value</th>
<th>Unit</th>
</tr>
</thead>
<tbody>
<tr>
<td>WBC</td>
<td>5.94</td>
<td>x10^3/μL</td>
</tr>
<tr>
<td>RBC</td>
<td>2.98</td>
<td>x10^6/μL</td>
</tr>
<tr>
<td>HGB</td>
<td>9.1</td>
<td>g/dL</td>
</tr>
<tr>
<td>HCT</td>
<td>26.9</td>
<td>%</td>
</tr>
<tr>
<td>MCV</td>
<td>90.3</td>
<td>fl</td>
</tr>
<tr>
<td>MCH</td>
<td>30.5</td>
<td>pg</td>
</tr>
<tr>
<td>MCHC</td>
<td>33.8</td>
<td>g/dL</td>
</tr>
<tr>
<td>PLT &amp;</td>
<td>24</td>
<td>x10^3/μL</td>
</tr>
<tr>
<td>RDW-SD</td>
<td>46.5</td>
<td>fl</td>
</tr>
<tr>
<td>RDW-CV</td>
<td>15.2</td>
<td>%</td>
</tr>
<tr>
<td>MPV</td>
<td>----</td>
<td>fl</td>
</tr>
<tr>
<td>NEUT</td>
<td>2.77</td>
<td>x10^3/μL</td>
</tr>
<tr>
<td>LYMPH</td>
<td>1.64</td>
<td>x10^3/μL</td>
</tr>
<tr>
<td>MONO</td>
<td>1.45</td>
<td>x10^3/μL</td>
</tr>
<tr>
<td>EO</td>
<td>0.00</td>
<td>x10^3/μL</td>
</tr>
<tr>
<td>BASO</td>
<td>0.08</td>
<td>x10^3/μL</td>
</tr>
<tr>
<td>RET</td>
<td>3.06</td>
<td>%</td>
</tr>
<tr>
<td>IRF</td>
<td>51.7</td>
<td>%</td>
</tr>
</tbody>
</table>

**WBC IP Message(s)**
- Monocytosis

**RBC/RET IP Message(s)**

**PLT IP Message(s)**
- PLT Abn Distribution
- Thrombocytopenia

**PLT Clumps?**

**Immature Gran?**
- Left Shift?
- NRBC?
- **Sysmex methods (R-3000/3500)**:
  - Sysmex use supravitally-fluorescence dye, **Auramine-O**.
  - *Forward-scatter* (measuring size) and *side-fluorescence intensity* (measure RNA’s content)
- **forward light scatter & side fluorescence** → **scattergram** → red cells, reticulocytes, platelets area

- **Reticulocytes area:**
  - LFR (low fluorescence ratio)
  - MFR (middle fluorescence ratio)
  - HFR (high fluorescence ratio)
  - IRF (Immature Retic.Fraction) = MFR + HFR

**LFR** : Heilmeyer III and IV

Flow-cytometry increase retics.count precision

Retic. 1% -- CV manl= 47.3%; automt = 6.4%
Retic. 9% -- CV manl= 27.2%; automt = 5.8%
- Bayer’s method (Advia-120) :

- Bayer uses Oxazine-750, a Nucleic-acid-binding dye.
- Erythrocyte changed into spherical-isovolumic.
- Measurement using 3 detectors:
  1. Low-angle-scatter (2-3°)
  2. High-angle-scatter (5-15°)
  3. Absorbance
- From these 3 detectors → 3 cytograms:

1. High-angle scatter vs Absorbans → LAC/MAC/HAC, IRF (MAC + HAC)
2. High-angle vs Low-angle-scatter (RBC map) → Reticulocyte’s indices
3. Volume vs Hb-concentration → CHr
- Automated Reticulocyte’s Parameters:

  - Retics # and %
  - HAC, MAC, LAC (Absorbance/Fluorescence Ratio)
  - IRF (Immature Retics Fraction = HAC + MAC)
  - Retic’s indices (MCVr, MCHr, CHCMr, CHr, RDWr, HDWr)
Fig. 11. CBC result (normal patient)
Fig. 12. CBC results (Reticulocytosis)

<table>
<thead>
<tr>
<th></th>
<th>Retic</th>
<th>%</th>
<th>#</th>
</tr>
</thead>
<tbody>
<tr>
<td>Neg RBC:</td>
<td></td>
<td>87.6</td>
<td>32015</td>
</tr>
<tr>
<td>Retic: H</td>
<td></td>
<td>12.4</td>
<td>519.2</td>
</tr>
<tr>
<td>L Retic:</td>
<td></td>
<td>72.3</td>
<td>3278</td>
</tr>
<tr>
<td>M Retic:</td>
<td></td>
<td>21.4</td>
<td>968</td>
</tr>
<tr>
<td>H Retic:</td>
<td></td>
<td>6.3</td>
<td>286</td>
</tr>
<tr>
<td>IRF-H:</td>
<td></td>
<td>6.3</td>
<td></td>
</tr>
<tr>
<td>IRF-M+H:</td>
<td></td>
<td>27.7</td>
<td></td>
</tr>
</tbody>
</table>

RTC Cells Acquired: 42910
RTC Cells Analyzed: 37486
RTC Gated Cells: 36547
Retic Count: 4532
Content of Retic’s Hb (CHr):

- Content of retic’s Hb never changed as long as the survival of Retics and red cells
- The CHr’s mean: 28.5 pg
- **CHr/CH ratio ± 1** (range: 0.96-1.03)
- The meaningful of CHr:
  - indicate Iron availability real-time
  - as a strong predictor for Iron Deficiency
  - Early indicator for Iron therapy in Iron Deficiency Anemia
- Reticulocyte’s Size/Volume (MCVr):

- Retic’s size drastically decreased along its maturation (Heilmeyer’s classification)

- MCVr/MCV ratio = 1.24 (constant in normal, microcytosis or macrocytosis)

- Stress Retics: MCVr/MCV ratio = >1.5-3

- Inverse MCVr/MCV ratio (<=1) → seen in Vit.B12 therapy’s response in Megaloblastic Anemia.
cell vol (fl)

120 fl

60 fl

28 g/dl 41 g/dl

→ Hb-concentr.(g/dl)
Fig. 13. Red Cell Analysis
Quantitation of:

- % Microcytic and % Macrocytic
- % Hypochromic and % Hyperchromic

Differentiate between $\beta$-thal trait and Iron Deficiency Anemia
- Differential Diagnosis of β-Thal trait and IDA:

  - **In β-Thal trait:**
    The *microcytosis* is more significant compared with mild hypochromia.

  - **In Fe Deficiency Anemia:**
    The *hypochromia* is more significant compared with mild microcytosis.
Ratio % M/H (Micro/Hypo)

- Ratio % M/H > 0.9 → β-Thal trait
- Ratio % M/H < 0.9 → IDA

- CHr combined with Ratio % M/H give stronger differentiation; Ratio % M/H < 0.9 with Low CHr give strong prediction for IDA
Clinical Applications of Retic’s Indices

- Retics = the first erythroid cell appeared in circulation and become mature red cell 24 hours later.

- Red cell morphological begin to change in the late-stage of Fe deficiency

- Retic.Indices reflects a real-time erythropoietic activities
- When Fe-store is low and erythropoiesis is decreased, red cell indicators are still normal. But marrow already release a new retic. with low Hb-content (low-CHr)
- Early detection of Functional Fe Deficiency is by measuring %-Hypochromic and CHr. %-Hypochromic reflects Hb concentration during 8-12 weeks.

- Fe-therapy responses have already seen from the CHr in 4 days (1-2 weeks) when normally it’s only seen from the increment of Hb after 1 month therapy.
Normal threshold 27 pg

Mature RBC’s
Reticulocytes

Haemoglobin content pg

DAY 0

DAY 4
(after IV Iron)

DAY 13

Haemoglobin content pg

The ADMS 129 protocol allows direct measurement of the haemoglobin content of the mature red cells (ER pg) and reticulocytes (ER pg).

These measurements can help optimize EPO and iron therapy.
HYPOCHROMIC MACROCYTES -
ARE THEY RETICULOCYTES?
- Hypochromic Macrocytic Cells?

- Reticulocyte?

- Dyserythropoiesis / Myelodysplastic syndrome / Sideroblastic anemia

- Fe-deficient megaloblastic anemia
Limitations

- Most dyes stain also other blood components containing DNA/RNA, so assessed as reticulocytes, i.e.: Howell-Jolly bodies, Cabot’s ring, Malaria parasites, white cell’s fragments in Leukemia

- Retics may “mature” during storage especially if not refrigerated
Thank You