QUANTITATIVE HEMATOLOGY: Automated Cell Counters
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WHY SHOULD YOU CARE?
• High volume: The CUMC Core Laboratory reports 440,000 CBCs and 193,000 differentials a year
• Many parameters measured
• Wide range of clinical applications

TALK OUTLINE
• Workflow in our hematology section
• Automated cell counters
  – Advantages of automated cell counters
  – Principles of automated cell counters
• Bayer Advia 2120
• Sysmex XE-2100
• CellaVision DM96

TYPES OF CELL COUNTERS
A) Fully Automated
B) Morphology-based

WHY USE AUTOMATED CELL COUNTERS??
DISADVANTAGES OF MANUAL CELL COUNTING:

- Cell identification errors in manual counting:
  - Mostly associated with distinguishing lymphocytes from monocytes, bands from segmented forms and abnormal cells (variant lymphocytes from blasts)
  - Lymphocytes overestimated, monocytes underestimated
- Slide cell distribution error:
  - Increased cell concentration along edges and in the feather edge, also bigger cells found there i.e. monocytes, eosinophils, and neutrophils
- Statistical sampling error

<table>
<thead>
<tr>
<th>Differential</th>
<th>Total number of cells counted</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>100</td>
</tr>
<tr>
<td>3</td>
<td>0-9</td>
</tr>
<tr>
<td>6</td>
<td>2-13</td>
</tr>
<tr>
<td>15</td>
<td>8-24</td>
</tr>
<tr>
<td>40</td>
<td>30-51</td>
</tr>
</tbody>
</table>

B.J. Bain: Blood cells. A practical guide

ADVANTAGES OF AUTOMATED CELL COUNTERS

- Objective (no inter-observer variability)
- No slide distribution error
- Eliminate statistical variations associated with manual count based on high number of cells counted
- Many parameters not available from a manual count, e.g. MCV, RDW…
- More efficient and cost effective than manual method:
  - Some cell counters can process 120-150 samples per hour
  - CAP assumes 11 minutes for a manual cell count

AUTOMATED CELL COUNTERS CAN PROVIDE:

- CBC: WBC, RBC, Hgb, Hct, PLT, RBC indices
- WBC Differential: 5 “normal” white cell types
- RDW, PDW, MPV
- Reticulocyte count
- Nucleated red cell count

All automated cell counters are screening devices. Abnormalities must be verified by a blood film, staining and scanning by an expert observer

WHO CARES HOW MANY SLIDES ARE MADE AND REVIEWED??

REVIEW RATE

- Whenever the automated cell counter flags a specimen, the technologist (or an automated system) has to
  - Retrieve the tube
  - Make a slide
  - Stain the slide
  - Review the slide
- Either release the results from the cell counter (“scan”) or perform a manual differential
- These steps consume time, labor, and money!!!
IMPORTANCE OF THE REVIEW RATE

- If it takes a technologist 10 minutes to prepare, label, stain, review, count, and result a slide, and
- If a technologist costs $25.00 per hour,
- It costs $4.00 to make a slide.
- If our laboratory’s review rate changes by 1% (= 2,000 differentials a year), we pay or save almost $10,000

PRINCIPLES OF AUTOMATED CELL COUNTERS

- Impedance (conductivity) system (Coulter)
- Optical system (H*)
- Both impedance and optical
- Selective lysis (e.g., lysis of red cells and counting of white cells)
- Special stains

1) ELECTRICAL IMPEDANCE METHOD
The “Coulter Principle”

- Cell counting and sizing is based on the detection and measurement of changes in electrical impedance (resistance) produced by a particle as it passes through a small aperture
- Particles such as blood cells are nonconductive but are suspended in an electrically conductive diluent
- As a dilute suspension of cells is drawn through the aperture, the passage of each individual cell momentarily increases the impedance (resistance) of the electrical path between two electrodes that are located on each side of the aperture

http://www.beckmancoulter.com

1) ELECTRICAL IMPEDANCE METHOD
The “Coulter Principle”

B.F. Rodak, Diagnostic Hematology, W.B. Saunders, 1995
2) OPTICAL METHOD
• Laser light is used
• A diluted blood specimen passes in a steady stream through which a beam of laser light is focused
• As each cell passes through the sensing zone of the flow cell, it scatters the focused light
• Scattered light is detected by a photodetector and converted to an electric impulse
• The number of impulses generated is directly proportional to the number of cells passing through the sensing zone in a specific period of time

2) OPTICAL METHOD
• The application of light scatter means that as a single cell passes across a laser light beam, the light will be reflected and scattered.
• The patterns of scatter are measured at various angles.
• Scattered light provides information about cell structure, shape, and reflectivity.
• These characteristics can be used to differentiate the various types of blood cells and to produce scatter plots with a five-part differential

HEMOGLOBIN MEASUREMENT
• Sample is diluted with cyanmethemoglobin reagent
• Potassium ferricyanide in the reagent converts the hemoglobin iron from the ferrous state (Fe+++) to the ferric state (Fe+++), to form methemoglobin,
• which then combines with potassium cyanide to form the stable cyanmethemoglobin
• A photodetector reads color intensity at 546 nm
• Optical density of the solution is proportional to the concentration of hemoglobin

TYPES OF ANALYZERS
• Smaller instruments: Measure erythrocytes, leukocytes, platelets (WBC, RBC, Hgb, Hct, MCV, MCH, MCHC, and platelets)
• Advanced cell counters: Add:
  – Red cell morphology information, RDW
  – Mean platelet volume
  – Leukocyte differential

ANALYZERS
• Abbot (http://www/abbott.com):
  – Cell-Dyn
• Siemens (Bayer) (http://www/bayerdiag.com):
  – Advia
• Beckman-Coulter (http://www/beckmancoulter.com):
  – STKS
  – Gen-S
• Sysmex (http://www/sysmex.com):
  – SE
TRENDS

- Current trends include attempts to incorporate as many analysis parameters as possible into one instrument platform, in order to minimize the need to run a single sample on multiple instruments (e.g. CD4 counts, smear preparation).
- Such instruments are being incorporated into highly automated combined chemistry/hematology laboratories, where samples are automatically sorted, aliquoted, and brought to the appropriate instrument by a robotic track system.

THE SIEMENS ADVIA 120/2120

RBC/PLATELET COUNT

- Parameters derived from light scatter in this channel:
  - MCV
  - RDW: correlates with anisocytosis
  - HDW: hemoglobin distribution width: correlates with degree of anisochromasia
  - Red cell Hb content
  - Mean platelet volume
  - Platelet distribution width: correlates with platelet anisocytosis
  - CHCM: cellular hemoglobin concentration mean
- Parameters calculated:
  - Hct: From MCV and RBC
  - MCHC: From Hb and Hct

MCHC

- Usual method:
  - Dividing the summation of the red cell volumes by the erythrocyte count
- H*1, H*2, Advia:
  - RBCs are sphered without altering their volume and lightly fixed
  - INDIVIDUAL RBC volume and hemoglobin concentration are determined by simultaneous measurement of light scatter at two different angles
  - Separate histograms for RBC volume and hemoglobin concentration are then derived

ADVIA® 120/2120: Cell-By-Cell RBC Analysis

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Conversion of Data to Cytogram and Histogram Using Mie Theory

**Hgb Concentration (HC)**
- 0 g/dL
- 28 g/dL
- 41 g/dL

**RBC Volume (fL)**
- 0 fL
- 60 fL
- 120 fL

**Hgb Content (CH)**
- 0 pg
- 100 pg

**Micro**
- 120 fL

**Hypo**
- 60 fL

**Hyper**
- 28 g/dL
- 41 g/dL

**NORMAL RED CELL CYTOGRAM**
- MCV: 85
- MCH: 29.6
- RDW: 13.0

**IRON DEFICIENCY – BLOOD SMEAR**
- Case 16
- MCV: 56
- MCH: 15.8
- RDW: 18.8

**IRON DEFICIENCY**
- Case 8
- MCV: 75
- MCH: 18.8
- RDW: 30.2

**Case 1**
- Iron: 24 (L)
- TIBC: 344
- Ferritin: 5 (L)

**Case 2**
- Iron: 36 (L)
- TIBC: 544 (H)
- Ferritin: 3 (L)

**THALASSEMIA TRAIT**
- Case 11
- RBC: 5.33
- MCV: 65
- MCH: 21.0
- RDW: 13.9

**Alpha Thalassemia Trait**
- Hemoglobin Electrophoresis: Normal pattern

**Case 18**
- RBC: 6.25
- MCV: 62
- MCH: 20.3
- RDW: 16.0

**Beta Thalassemia Trait**
- Hemoglobin Electrophoresis: Hb A₂: 5.2 (H)

**HEMOLYSIS**
- Case 10
- MCV: 72
- MCH: 23.0
- RDW: 14.9

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- Case 10
- MCV: 72
- MCH: 23.0
- RDW: 14.9

**SPHEROCYTE**
WHAT IS THIS??????????

RBC: 3.36
MCV: 85
MCH: 28.9
RDW: 21.7

Case 13

RED CELL AGGLUTINATES

RBC: 3.61
MCV: 78
MCH: 26.9
RDW: 14.2

Case 14

COLD AGGLUTININ

RBC: 3.36
MCV: 85
MCH: 28.9
RDW: 21.7

Warm Sample

RBC: 3.61
MCV: 78
MCH: 26.9
RDW: 14.2

Case 14

USE OF RBC PARAMETERS IN CLINICAL PRACTICE

• Average life span of a red cell: 120 days;
• Every day, only approximately 1% of the red cell mass is renewed
• Therefore, total red cell parameters are NOT ideal as early indicators of erythropoetic change;
• In contrast, reticulocytes only circulate for 1 day; reticulocyte parameters are therefore better suited as early indicators of erythropoetic change:

USE OF RETICULOCYTE PARAMETERS IN CLINICAL PRACTICE

• Based on uptake of a nucleic acid dye, oxazine 750, by RNA
• Cells that have taken up the dye absorb more light than mature red cells that have not taken up the dye, and can thus be counted
• Size and Hb content of reticulocytes are measured by light scatter
ADVIA® 120 RETICULOCYTE ANALYSIS

High angle detector (9° - 15°)
Low angle detector (2° - 3°)

670nm Laser Diode
Oxazine 750 RNA Stain

Absorbance RNA Content

RETICULOCYTE PARAMETERS AVAILABLE ON THE ADVIA® 120

- Reticulocyte count
- MCVr
- CHCMr
- CHr (mean hemoglobin content of reticulocytes)
- RDWr
- HDWr

CORRECTING IRON DEFICIENCY

CHr: 35%
CHm: 27%
MCV: 82
MCH: 26.3
RDW: 26.2

Case 9

CLINICAL APPLICATIONS OF RETICULOCYTE PARAMETERS

- Guidance of iron and EPO therapy in hemodialysis patients
- Diagnosis of iron deficiency in patients with inflammation or chronic disease
- Diagnosis of iron deficiency in early childhood

(Clin Chem 2003;49:1573)

CORRECTING IRON DEFICIENCY


<table>
<thead>
<tr>
<th>Day 0</th>
<th>Day 4</th>
<th>Day 10</th>
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<tbody>
<tr>
<td>MCV</td>
<td>59</td>
<td>66</td>
</tr>
<tr>
<td>MCH</td>
<td>14.1</td>
<td>15.8</td>
</tr>
<tr>
<td>CHr</td>
<td>16.2</td>
<td>26.1</td>
</tr>
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</table>

WHITE CELL DIFFERENTIAL

- The Advia can identify six white cell types:
  - Neutrophil
  - Eosinophil
  - Basophil
  - Monocyte
  - Lymphocyte
  - Large unstained cell
- A lobularity index (left shift indicator) is also reported
WHITE CELL DIFFERENTIAL

The ADVIA uses TWO separate channels to determine the white cell count:
– 1) Peroxidase channel
– 2) Basophil/lobularity channel

PEROXIDASE CHANNEL

• Specimen is diluted with peroxidase reagents, red cells and platelets are lysed
• Dark precipitate forms in cells containing peroxidase:
  – Neutrophils, eosinophils, monocytes
• Lymphocytes, basophils, large unstained cells contain no peroxidase and therefore do not stain
• A tungsten-based optical system is used to measure:
  – Individual impulses are counted to determine WBC
  – Stain intensity (absorbance) for each cell
  – Cell size (by forward light scatter)

PEROXIDASE CHANNEL

• Cells are plotted on a scattergram: scatter (size) on y-axis and peroxidase activity (staining intensity) on the x-axis
• Each cell is classified based on this information
• In this channel, basophils are classified with lymphocytes

PEROXIDASE CHANNEL

• Lymphocytes: Small, unstained cells
• Larger atypical lymphocytes, plasma cells, and some blasts: “large unstained cells”
• Eosinophils: Strongest peroxidase activity and appear smaller than neutrophils
• Neutrophils are large and have moderate peroxidase activity
• Monocytes have somewhat less peroxidase staining and are therefore to the left of the neutrophils and right of the LUCs.

WBC PEROXIDASE CHANNEL

Patient Peripheral Blood

• WBC Count: 6,760 cells/µL
• 1.6% Neut
• 21.5% Lymph
• 69.5% LUC
• 66.0% PMN
• 33.7% MN
• 3+ ATYPS Flag
• 1+ MPO Deficiency Flag

MYELOPEROXIDASE DEFICIENCY

Normal Peripheral Blood

Patient Peripheral Blood

ADVIA 120 Results
BASOPHIL/LOBULARITY CHANNEL

- Surfactant and phthalic acid are added to lyse the RBCs and strip away the cytoplasmic membrane from all leucocytes except the basophils.
- Laser light signal from a low-angle forward scatter plot identifies basophils, which, due to their retained cytoplasm, are larger than other cells.
- A high-angle detector is sensitive to the shape and structure (lobularity and density) of remaining nuclei.

FLAGS GENERATED BY COMBINATIONS OF THE CHANNELS

In addition to numerical data and various flags generated by the peroxidase and the basophil channels individually, other flags are generated by comparison of the channels:

- Discrepancy of the two WBC counts: Can be due to non-lysis of RBC in peroxidase channel.
- Flag for immature granulocytes: Presence of more peroxidase-positive cells than expected from the number of PMN in basophil channel.
- NRBC flag: Excess of cells in PMN in comparison with sum of eosinophils and neutrophils in peroxidase channel.

THE SYSMEX XE-2100
THE SYSMEX XE-2100

- Uses both impedance and flow cytometry
- Throughput: 150 samples/hour
- Provides 32 parameters, including reticulocyte and NRBC counts
- Uses an optical fluorescent platelet count when the impedance count may be unreliable
- Unique features:
  - Can enumerate, not just flag for, immature granulocytes (metamyelocytes, myelocytes, promyelocytes)
  - Immature platelet fraction

Arch Pathol Lab Med 2001; 125:391-396

THE SYSMEX XE-2100

Flow Cytometry (Forward light scatter, side light scatter, side fluorescence) for:
- WBC differential, NRBC, reticulocytes, optical platelets
- Radio frequency (RF) and direct current (DC) resistance for:
  - Immature granulocytes

Sysmex J Int 9:31-44, 1999

HEMOGLOBIN MEASUREMENT

- Red cells are lysed, hemoglobin is converted into sodium lauryl sulfate (SLS)-methemoglobin:
  - Short reaction time
- Absorbance at 555 nm is measured, and concentration of hemoglobin is calculated
  - Good correlation with reference method

Sysmex XE-Series Pre-Training Guide

RED CELL AND PLATELET COUNTS

Red cells and platelets are both counted by the electric impedance method.
In samples with large platelets or small RBCs or RBC fragments, platelet counts by the light scattering method are used instead.

Sysmex J Int 9:31-44, 1999

WBC/BASO CHANNEL

- Red cells are lysed, degranulation of basophils is selectively suppressed
- Forward and side scatter are used to obtain a WBC and basophil count

Arch Pathol Lab Med 2001; 125:391-396

4-DIFF CHANNEL

- Red cells are lysed, DNA and RNA of WBCs are stained with fluorescent dye
- Side fluorescence and side scatter are used to obtain a four-part differential

Arch Pathol Lab Med 2001; 125:391-396
**Immature Granulocyte Count**

- Includes promyelocytes, metamyelocytes, myelocytes, but NOT bands or blasts
- A lysing reagent causes disruption of mature WBC membranes, while immature myeloid cells with low membrane lipid content remain intact.

**IMI CHANNEL**

- Red cells are lysed, a lysing agent which disrupts the cell membranes of MATURE WBCs only is used.
- Analysis by radiofrequency and direct current impedance detection

**ABNORMAL CELLS IN THE DIFF AND IN THE IMI CHANNEL**

**Detection of Stem Cells in the IMI Channel**

**NRBC CHANNEL**

- Red cells are lysed, WBCs are stained with fluorescent dye; NRBC nuclei are shrunk and slightly stained

**Immmature Granulocyte Count**

- Correlation coefficient with visual count: 0.81
- Prediction of infection:
  - At 90% specificity, sensitivity of 35-40%
  - At 90% sensitivity, specificity of 20%
  - Better predictor of sepsis than WBC; comparable to ANC

**Arch Pathol Lab Med 2001; 125:391-396**

**Lab Hematol 2004; 10:200-205**
Reticulocyte Count
- RBCs are stained with a fluorescent dye
- Fluorescent intensity (DNA/RNA content) and forward light scatter (cell size) are plotted to distinguish reticulocytes from mature RBCs, platelets, and WBC.
- Also measured in this channel:
  - “Optical” platelet count
  - Forward light scatter of mature RBCs (RBC-Y) and reticulocytes (RET-Y)

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Immature platelet fraction
- Fluorescent dye stains the platelet membrane and granules
- Applications:
  - Higher values in patients with ITP, DIC; normal values in patients with decreased platelet production
  - An increase in immature platelet fraction indicates impending platelet recovery; may precede recovery by several days; may allow optimal perfusion of platelet concentrates

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### Immature platelet fraction

<table>
<thead>
<tr>
<th>Normal</th>
<th>TIPREC</th>
</tr>
</thead>
<tbody>
<tr>
<td>Retic count</td>
<td>3-4%</td>
</tr>
<tr>
<td>Ht</td>
<td>35-45%</td>
</tr>
</tbody>
</table>

- The forward light scatter of the reticulocytes; correlated with their size and content
- Correlates very well with CHr (r=0.94)
- May provide equivalent information to CHr in the monitoring of iron therapy

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**RET-Y**

- The forward light scatter of the reticulocytes; correlated with their size and content
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- May provide equivalent information to CHr in the monitoring of iron therapy

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**WBC differential ordered**

- Automated differential performed
  - No flags
  - Flag(s)
  - Automated differential is reported
  - Slide made, labeled, stained, reviewed
  - Review with Microscope
  - Review with CellaVision
  - Automated differential is reported
  - Morphology-based differential is reported

**WHAT DOES THE TECHNOLOGIST USE TO REVIEW THE SLIDE?**

**THE CELLAVISION DM96**
THE CELLAVISION DM96

- An automated image analysis system for peripheral blood smears
- Barcode-labeled, stained slides are placed into a magazine
- Instrument scans slides at low power, identifies potential WBCs, and takes digital images at high magnification
- Images are analyzed by an artificial neural network and pre-classified according to white cell class

THE CELLAVISION DM96

- The WBCs and their suggested classifications are presented to the user on a computer display for confirmation or re-classification
- The system also provides functionality for the review of RBC and platelet morphology and for estimation of the platelet count

COMPARISON OF THE CELLAVISION WITH DIRECT MICROSCOPY

- Many cases were “false positive” with the CellaVision: The technologists reported cells with the CellaVision which they did not see with the microscope
- The discrepancies involved mainly very small populations of cells (<5%)
- For example, on 13 out of 96 blood smears the technologist reported NRBCs with the CellaVision, but NOT with the microscope
WHY DO TECHNOLOGISTS SEE AND REPORT LOW-FREQUENCY CELLS WITH THE CELLAVISION AND NOT WITH THE MICROSCOPE?

EXPLANATION: YOU CAN NOT “SKIP” CELLS WITH THE CELLAVISION!!!

ANY QUESTIONS???