Reticulocyte Count

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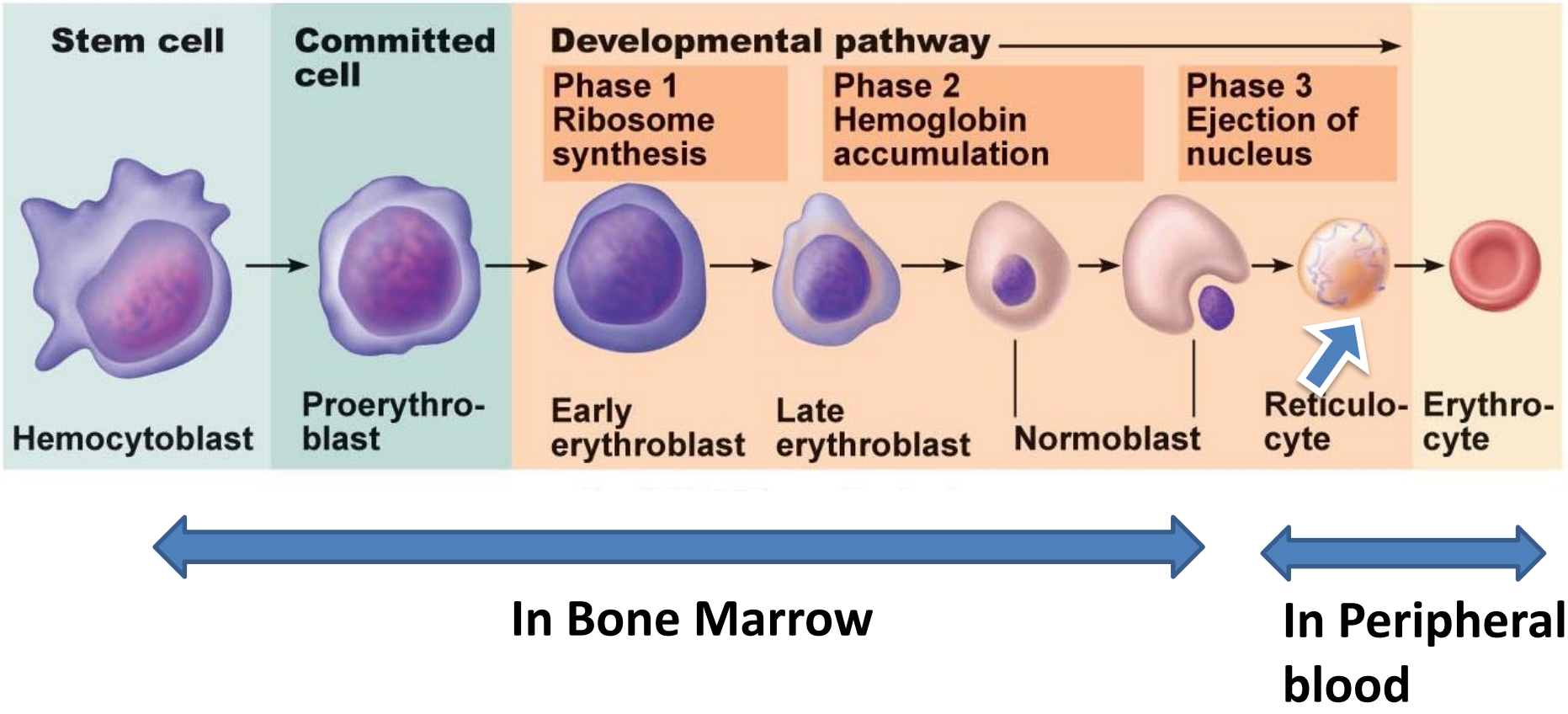
What are Reticulocytes?

Reticulocyte are immature, non-nucleated erythrocytes retaining a small network of basophilic organelles, consisting of RNA and protoporphyrin .

Source of Reticulocytes

Erythropoiesis



Morphological definition

- **Stage 0**:Orthochromatic normoblast
- **Stage I:**Dense coherent reticulum in non-nucleated cell
- **Stage II**: Extended network of loose reticulum
- **Stage III**: Scattered granules with residual reticulum
- **Stage IV** : Scattered granules

The maturation stages of the reticulocyte were described by **Heilmeyer** in **1932**

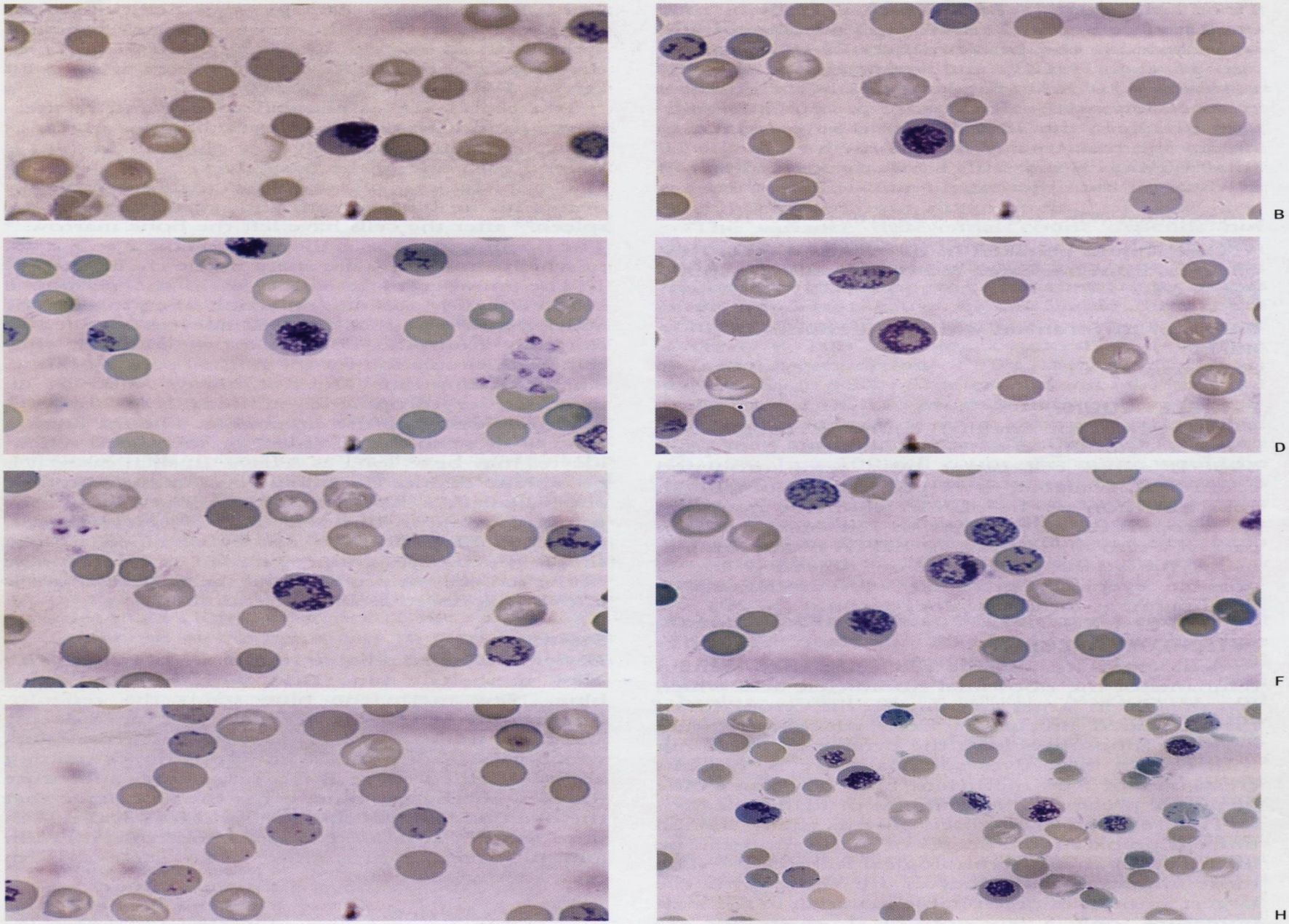


Fig. 3.4 Photomicrographs of reticulocytes showing stages of maturation. A and B, most immature (group I); C and D, intermediate (group II); E and F, later stage itermediate (group III); G, most mature (group IV); H, haemolytic anaemia, stained supravitally by new methylene blue.

Why?

- The number of reticulocytes in the peripheral blood is a **fairly accurate reflection of erythropoietic activity**, assuming that the reticulocytes are released normally from the bone marrow and that they remain in circulation for the normal time period.

- The enumeration of reticulocytes provides a simple, effective means to determine red cell production and regeneration

Implications

- **Anemia workup** (peripheral destruction vs failure of production)
- **Response to therapy** (iron, vitamin B-12, folic acid supplementation)
- **Bone marrow recovery**(after bone marrow transplantation or intensive chemotherapy)

Specimen collection

- **Venous or capillary blood**

- **EDTA is the anticoagulant of choice** for full blood estimations, it is also the most suitable one for reticulocyte analysis, reducing the need for a second specimen. However, any anticoagulated blood is suitable.

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Specimen Storage

- Promptly after the collection of the blood specimen
- At R.T- within six hours after blood collection.
- Apparent **in vitro maturation and subsequent disappearance** of some of the reticulocytes.
- This maturation is both time and temperature-dependent.
- If sample analysis is delayed, the sample should be refrigerated. Samples stored at 2 to 6 ^{o}c may be stable for up to 48 hours

NCCLS guidelines H44-A2 Vol. 24 No. 8,2004

Reticulocyte count

Manual method- supravital dye-light microscopy

fluorescent dye-fluorescent microscopy

Phase contrast microscopy

Automated cell counters

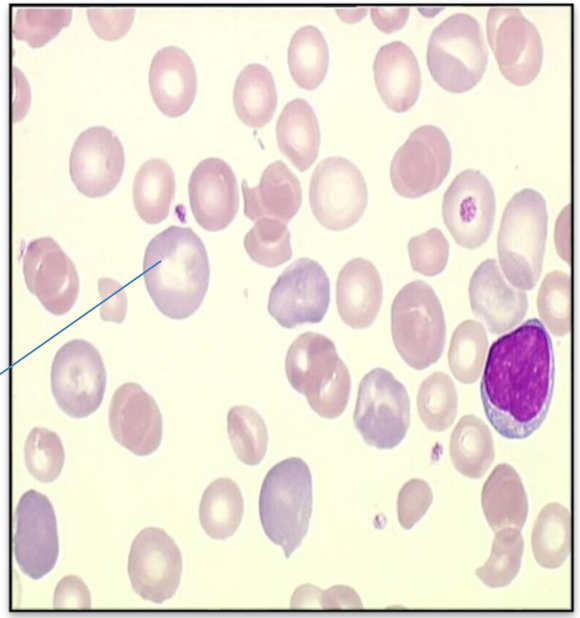
Supravital staining

✵ **PRINCIPLE:**

- RNA within the ribosomes reacts with basic dyes such as **azure B, brilliant cresyl blue or New methylene blue** to form a blue precipitate

- This reaction only occurs in 'vital' unfixed preparations

✵ On Romanowsky stains: Reticulocytes appear as polychromatophilic (grey/blue) cells



Staining solution

1% Brilliant cresyl blue or 1% New methylene blue or Azure B dye.

Dissolve 1gm of BCB / NMB/Azure B dye in 100ml of 3% trisodium citrate-saline/ 100ml of iso-osmotic phosphate buffer (pH 6.5).

- Check pH using pH meter
- Filter with Whitman's filter paper No.1
- Keep in dry bottle
- Stable for 4 – 6 weeks

Method

- Incubate the mixture at 37^0C in water bath for 15-20 min.

- Resuspend the red cells by gentle mixing.

- Make films on glass slides in the usual way.
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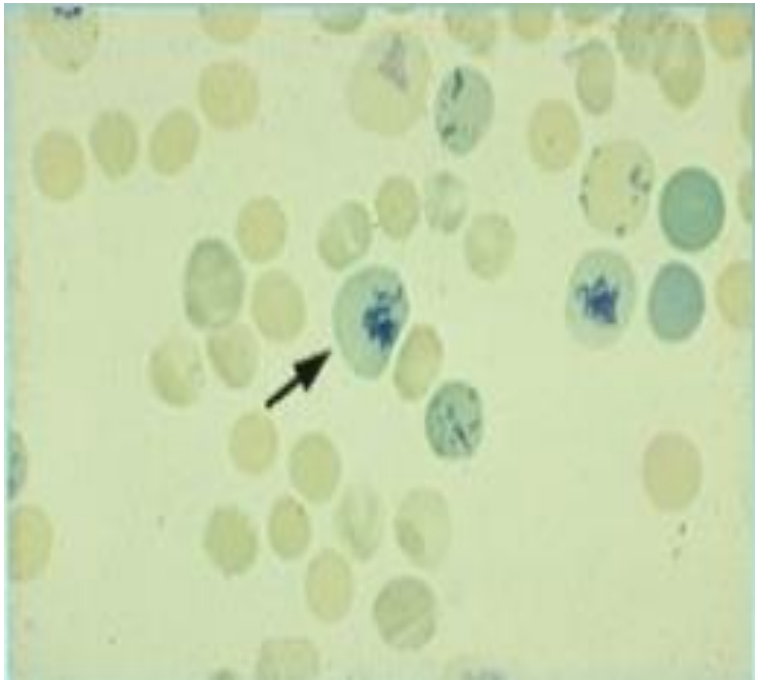
- When dry, examine the films without fixing or counterstaining.

Manual reticulocyte count



Counting of Reticulocyte

- There are two commonly used methods for counting reticulocytes.

— Standard counting procedure

— Miller Reticle procedure

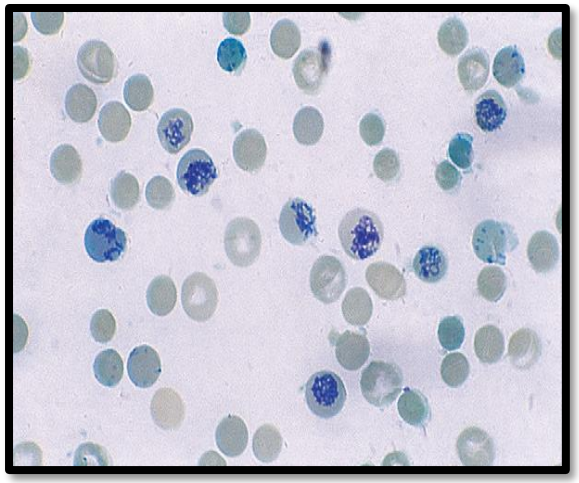
- Standard counting procedure

✓ Dry smear is examined under oil immersion.

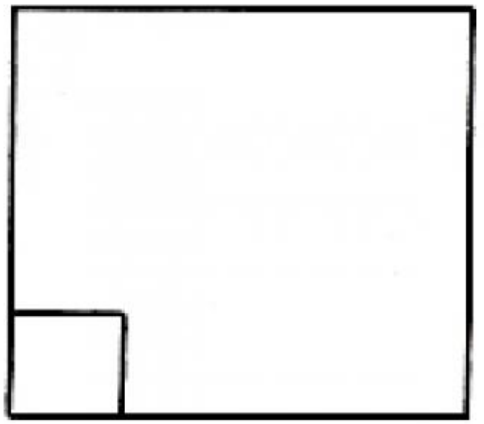
✓ Total of 1,000 RBCs are counted.

✓ Number of reticulocytes are recorded per 1,000 RBCs.

✓ Calculate the percentage of Reticulocyte and absolute reticulocyte count.



Counting of Reticulocyte Count



- Miller Reticle Procedure

✓ Dry smear is examined under oil immersion.

✓ A eyepiece with a **Miller reticle** can be used.

✓ It is two squares. One large square with a smaller square in the corner.

✓ For each field of view the retics are counted in the whole large square (including the small).

✓ All RBCs lying within the small square are counted including reticulocytes and that number is recorded as red cells.

✓ This is repeated until 100 to 200 RBCs are counted in the small squares.

✓ The disc was invented by Dr. JW Miller of the National Institutes of Health (NIH).

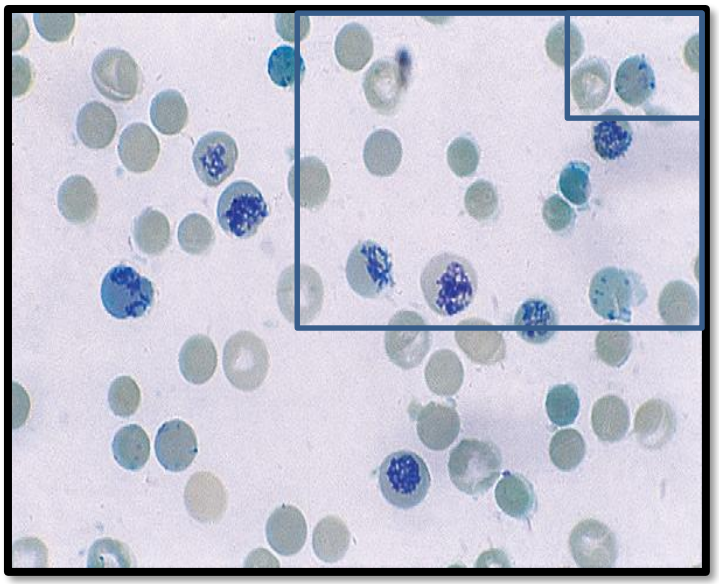
✓ Recommended by both NCCLS and ICSH

Counting of Reticulocyte Count

- Miller Reticle Calculating Procedure

- The formula is different than the standard because of the Miller reticle squares used.

✓ $\frac{\text{\# of reticulocytes counted}}{\text{Total \# RBCs counted} \times 9} \times 100 = \%\ \text{Retics}$



Reporting Results

- **Reticulocyte count Percent:**
- **Absolute Reticulocyte Count (ARC):** is the actual number of reticulocytes in 1L of whole blood. This is calculated by multiplying the reticulocytes % by the RBCs count and dividing by 100.

- **Corrected Reticulocyte Count** is calculated based on a normal hematocrit of 45%.

- **Reticulocyte Production Index (RPI) =** Corrected retic count (%) / # Days (Maturation time)

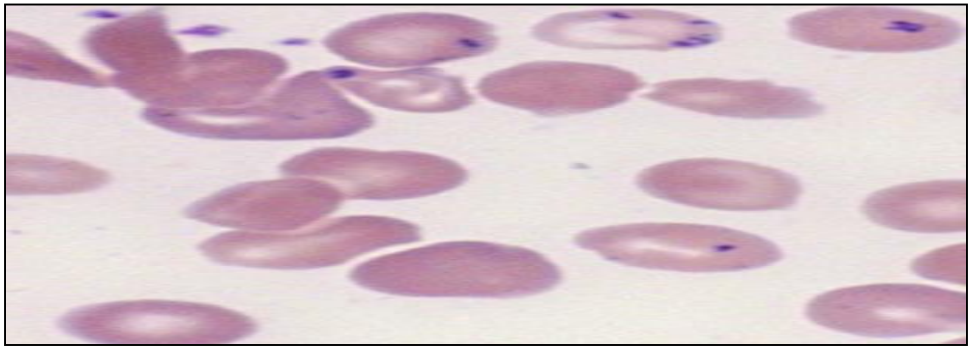
WHAT CAN AFFECT THE TEST

- Taking medicines, such as levodopa, corticotropin, azathioprine (Inurn), chloramphenicol (Chloromycetin), dactinomycin (Cosmegen), medicines to reduce a fever, medicines to treat malaria, and methotrexate and other cancer chemotherapy medicines.
- Taking sulfonamide antibiotics (such as Bactrim or Septra)
- Getting radiation therapy
- Being pregnant
- Having a recent blood transfusion

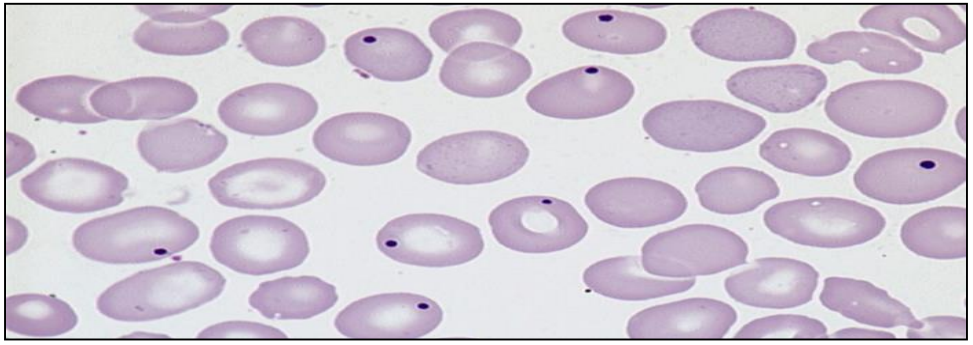
SOURCES OF ERROR

1. A refractile appearance of erythrocytes should not be confused with reticulocytes.
2. Filtration of the stain is necessary when precipitated material is present which can resemble a reticulocyte.
3. Erythrocyte inclusions should not be mistaken for Reticulocytes.
   - Howell-Jolly bodies appear as one or sometime two, deep-purple dense structures.
   - Heinz bodies stain a light blue-green and are usually present at the edge of the erythrocyte.
   - Pappenheimer bodies are **more often confused** with reticulocytes and are the most difficult to distinguish. These purple-staining iron deposits generally appear as several granules in a small cluster. If Pappenheimer bodies are suspected, stain with Wright- Giemsa to verify their presence.
   - Hemoglobin H inclusions will appear as multiple small dots in every cell.

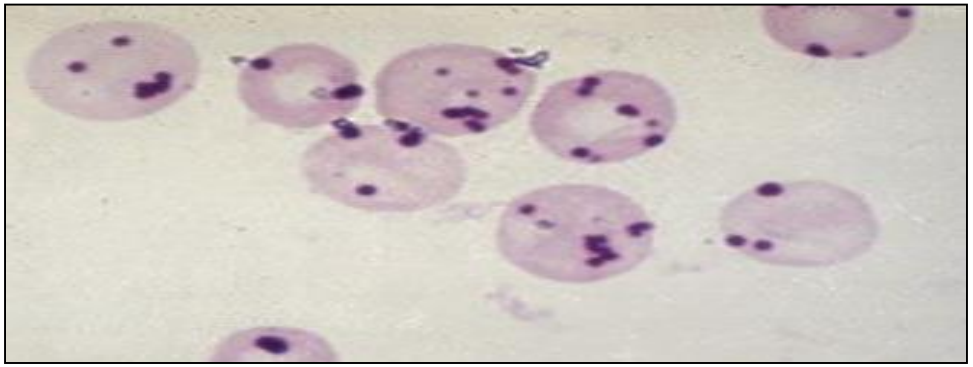
- Pappenheimer bodies



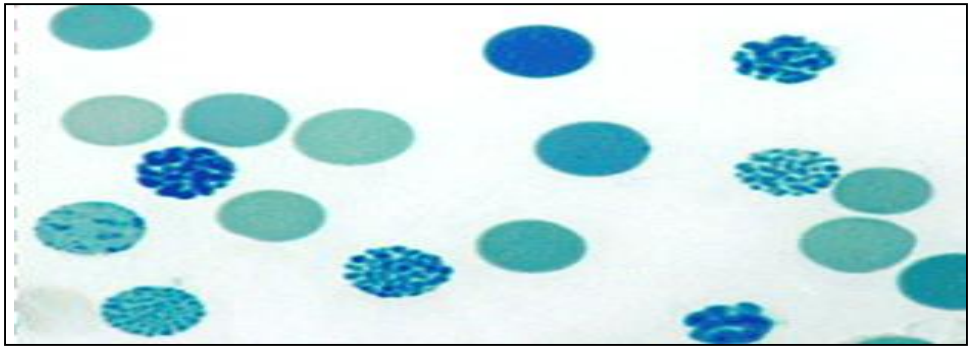
- Howell-Jolly bodies



- Heinz bodies



- Hemoglobin H inclusion

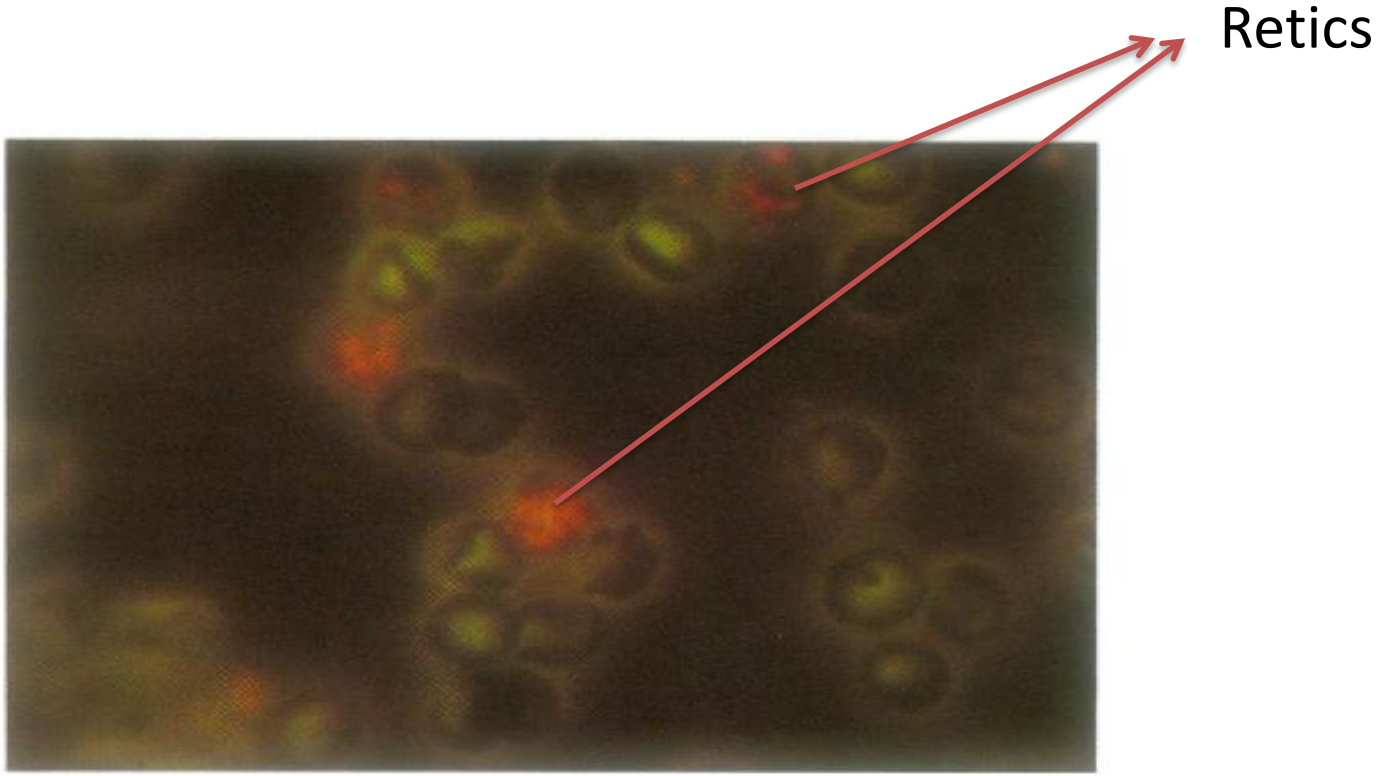


4. Falsely decreased reticulocyte counts can result from under staining the blood with new methylene blue. Be sure the stain/blood mixture incubates the full 20 minutes.
5. There is high degree of inaccuracy in the manual reticulocyte count owing to error (± 2%) in low counts and ± 7% in high counts) and a lack of reproducibility because of the inaccuracy of the blood film. This inaccuracy has been overcome by the use of automated instruments using flow cytometry.
6. If no reticulocytes are observed after scanning at least two slides, report "**none seen**".

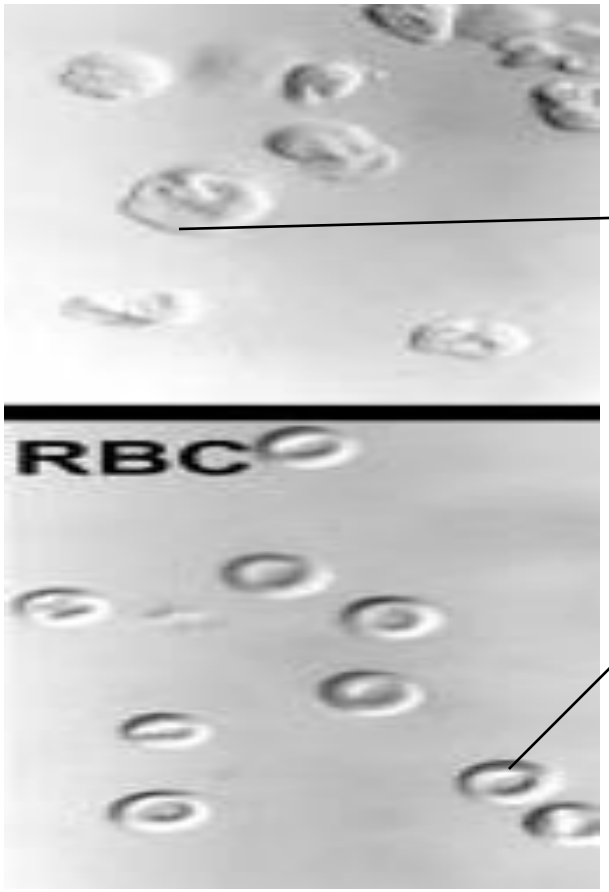
Fluorescence Method

- Manually by fluorescence microscopy.
- Add 1 volume of acridine orange solution (50 mg/100 ml of 9 g/l NaCl) to 1 volume of blood.
- Mix gently for 2 min; make films on glass slides, dry rapidly, and examine by a fluorescent microscope.
- RNA gives an orange–red fluorescence, whereas nuclear material (DNA) fluoresces yellow.
- The amount of fluorescence is proportional to the amount of RNA,
- The brightness and colour of the fluorescence fluctuates and the preparation quickly fades when exposed to light; also, it requires a special fluorescence microscope.
- It is thus not suitable for routine use for reticulocyte counting.

Fluorescence method



Phase contrast microscopy



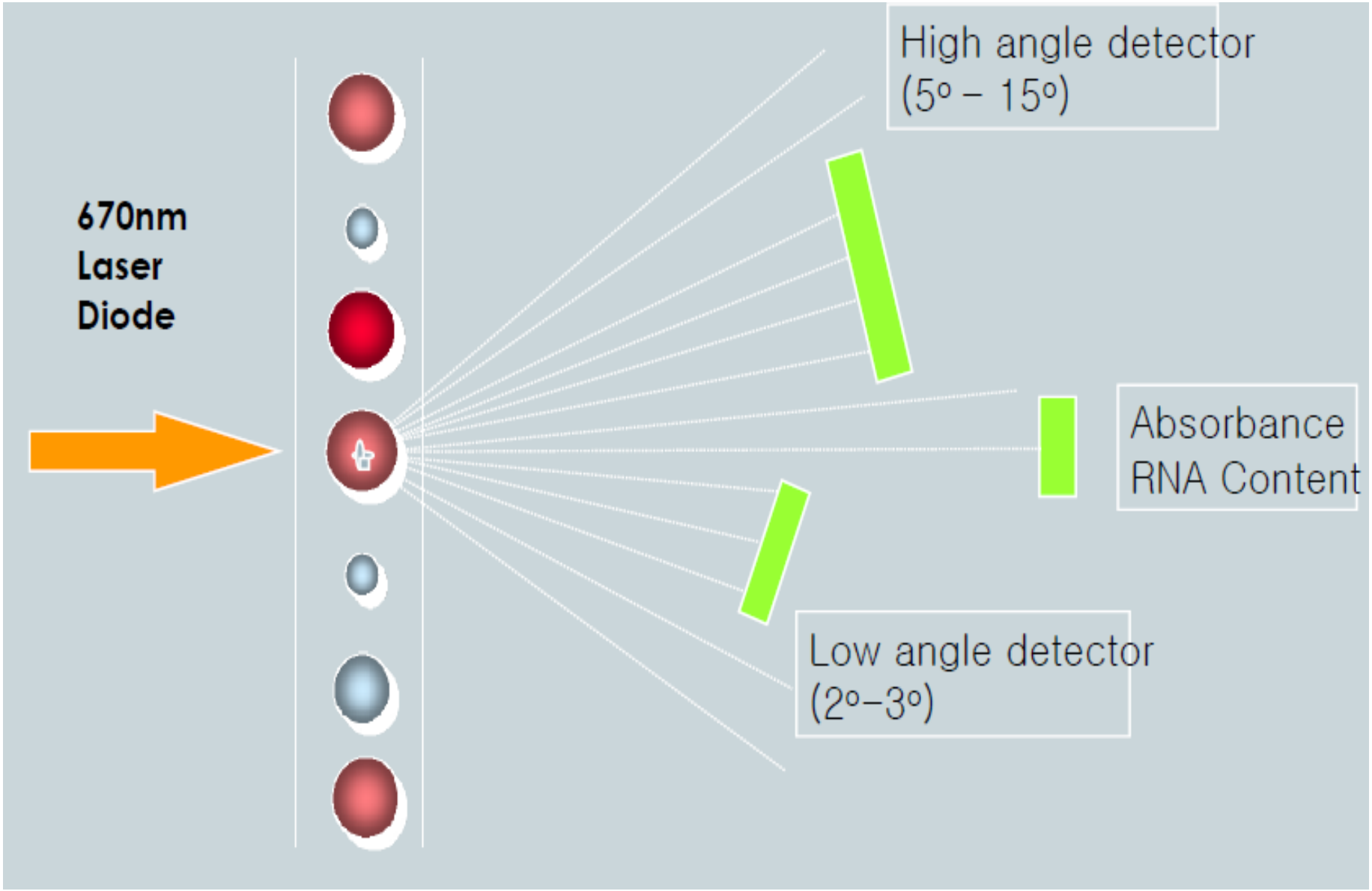
Reticulocyte

Mature RBC

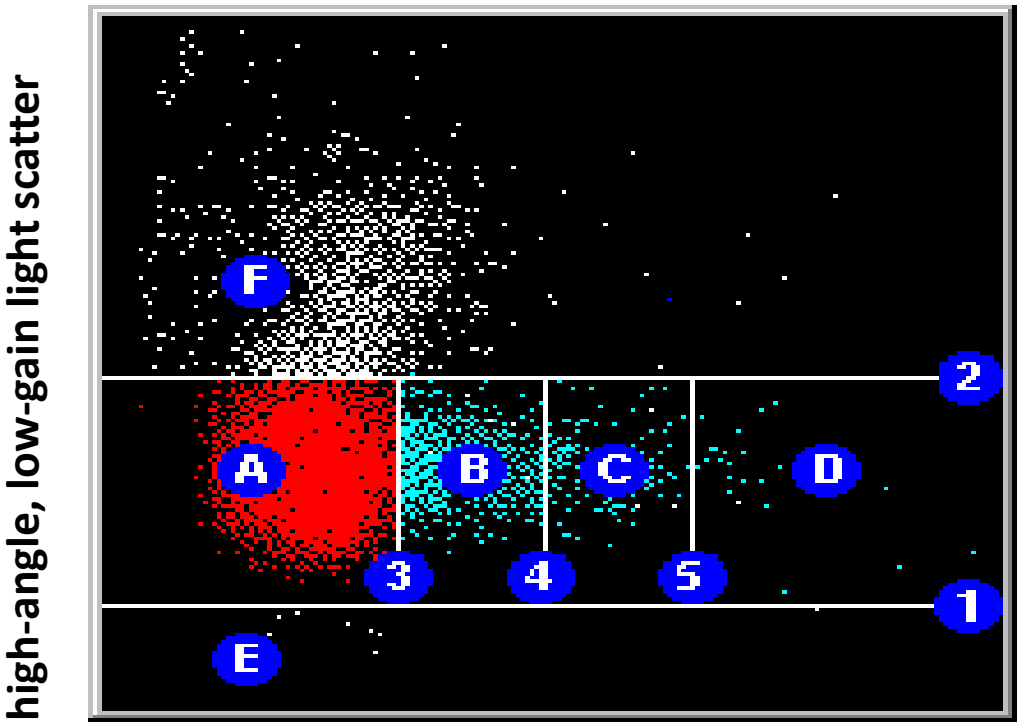
Analytic Methods of Reticulocyte Count

Analyzer	Dye	Technology
ADVIA 2120i	Oxazine 750	Absorbance
CELL DYN 4000	CD4K 530	Fluorescence
DXH-800	New methylene blue	Light scattering
SE 9500 RET	Auramine O	Fluorescence
HORIBA	Thiazole orange	Fluorescence

Reticulocyte Analysis



RETIC Scatter Abs Cytogram



1 RTC Platelet threshold
2 RTC Coincidence threshold
3 RTC threshold
4 Low/Medium RTC threshold
5 Medium/High RTC threshold

A Mature RBCs
B Low absorption retics
C Medium absorption retics
D High absorption retics
E Platelets
F Coincidence events

Calibration Procedure

Setting the RETIC gain factor provides sufficient accuracy of the %RETIC result

Do not change the factory-set %RETIC calibration factor of 1.00.

Manual counting method other than those recommended by NCCLS or ICSH, or laboratories using certain flow cytometric Methods
Use the calibration procedure to obtain agreement with the other method.

Quality Control

ADVIA TESTpoint Reticulocyte Controls.
Controls are intended to be integrated into a clinical laboratory's own quality control program and procedures.

New Parameters with Automated analyzer

Immature Reticulocyte Fraction (IRF)

population of reticulocytes most recently released from the bone marrow contains the highest concentration of RNA

The IRF is a promising new hematology parameter that needs further integration into clinical practice with cooperation between manufacturers, clinicians, laboratories, and standards-setting organizations

formerly termed reticulocyte maturity index (RMI)
effectively replaced the previous practice of adjusting the reticulocyte count based upon the level of anemia

Immature Reticulocyte Fraction (IRF): Clinical Utility in Medical Practice

- Monitor BM or Stem Cell Regeneration post-BMT or ChemoRx
- Monitor Renal Transplant Engraftment (Epo production)
- Monitor Neonatal Transfusion Needs
- Monitor Anemia Therapy
- Monitor EPO Therapy: Renal Failure, AIDS, Infants, MDS
- Monitor Bone Marrow Toxic Insults from drugs
- Prognostic in Anemia of AIDS and Prematurity
- Timing for Stem Cell Harvests following Growth Factor or Cytotoxic Drug Therapy
- Detection of Aplastic Crisis in Hemolytic Anemias
- Diagnosis and monitoring of aplastic anemia
- Evaluate Normochromic Anemias of Various Etiologies
- Detection of Occult or Compensated Hemorrhage or Hemolysis
- Classification of Anemias

Patterns of IRF and Retic counts in Anemia

Clinical Condition	Retic Ct	IRF
• Aplastic anemia/crisis	Low	Low
• BM regeneration	Low	High/WNL
• Iron deficiency	Low/WNL	High
• Thalassemia	WNL/high	WNL/high
• Hemolytic anemia/Blood loss	High	High

Reticulocyte Hemoglobin Content (CHr)

The CHr is the mean of cellular hemoglobin content (CH) histogram for the reticulocyte population.

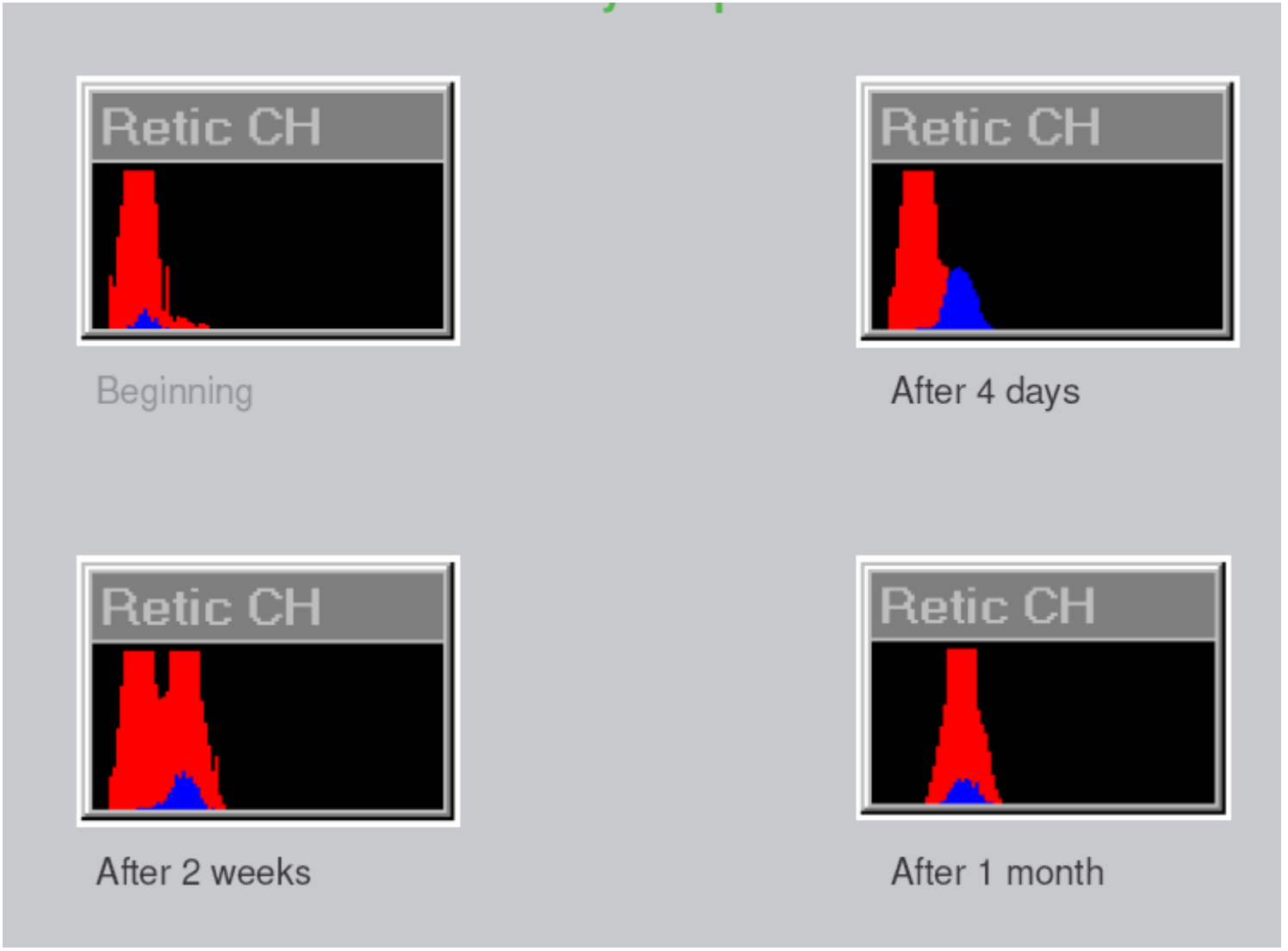
An early indicator of functional iron deficiency.

Functional iron deficiency has been shown to occur when erythropoietin is employed in the treatment of anemia associated with end stage renal failure and other diseases.

CHr is more sensitive and specific in the detection of functional iron deficiency than traditional methods for the detection of iron deficiency

Used in managing the iron requirements for therapy

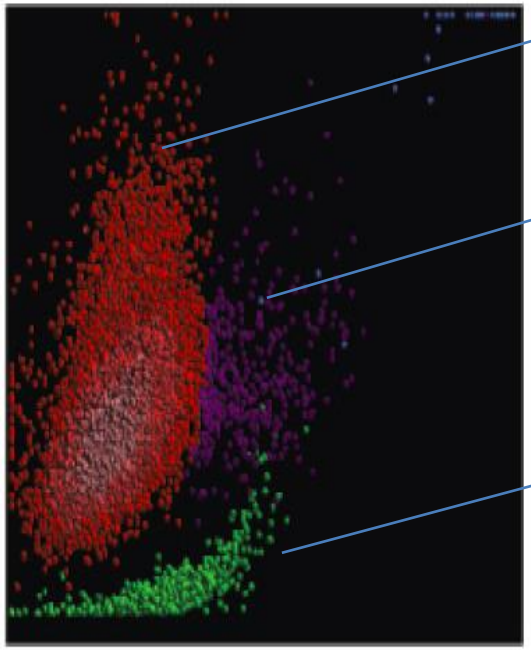
Erythropoietin treatment



Reticulocyte Method: VCS technology

- Reticulocyte analysis uses new methylene blue stain to identify reticulated red cells by precipitating the residual RNA.
- A portion of the blood sample is diluted and treated with a hypo-osmotic ghosting solution to clear the red cells of hemoglobin while preserving the stained RNA contained within reticulocytes.
- Nucleated cells and platelets are maintained in predictable states

Reticulocyte Method



Volume vs. LLSn

The dataplot shows mature red cells and Reticulocytes.

Cell volume (V)—Y-axis and Linear light scatter (LLSn) —x-axis

Advantages of automated Retic count

- Objective (no inter-observer variability)

- No slide distribution error , speed and reagent stability

- Eliminate statistical variations associated with manual count based on high number of cells counted

- Many parameters not available from a manual count, e.g. MCVr, CHr etc.

- More efficient and cost effective than manual method:
  – Some cell counters can process 74 samples per hour

Limitations of the Procedure

Samples with extremely elevated counts, the reticulocyte method may give reticulocyte counts that differ significantly from NCCLS manual counts. Extremely elevated counts are often seen in samples that contain sickled cells or nucleated RBCs.

Samples having the following cell types or conditions may interfere with the ADVIA 120 Reticulocyte method:
Malarial parasites
Pappenheimer Bodies
Howell-Jolly Bodies
Heinz Bodies
Inclusions that give rise to basophilic stippling
Macrothrombocytes (giant platelets)
Megaloblastic anemia
Other rare, poorly characterized red cell anomalies

Take Home Message

•Along with CBC, and peripheral blood smear examination, reticulocyte count is one of the first tests to be performed while investigating anaemia.

•Reticulocyte count is a simple, easy to perform and inexpensive test

•Evenly smeared slide and appropriate staining is essential for proper interpretation.

•Automated methods are faster and gives more promising parameters with precision.

References:

- Dacie and Lewis –Practical Hematology, edition 2012

- Siemens -Advia 2120i Manual

- Beckman coulter -DXH800 Manual

Thank You