Romanowsky & Cytochemical stains

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Contents

- Preparation of peripheral blood film
- Romanowsky & special stains in hematology
- Principle of staining
- Staining artifacts
- Troubleshooting

Preparation of blood film

- Blood films are prepared from fresh blood with no anticoagulant or from **EDTA/ Citrate** (anticoagulated) blood.

- Avoid- Heparinised/ K3 EDTA blood

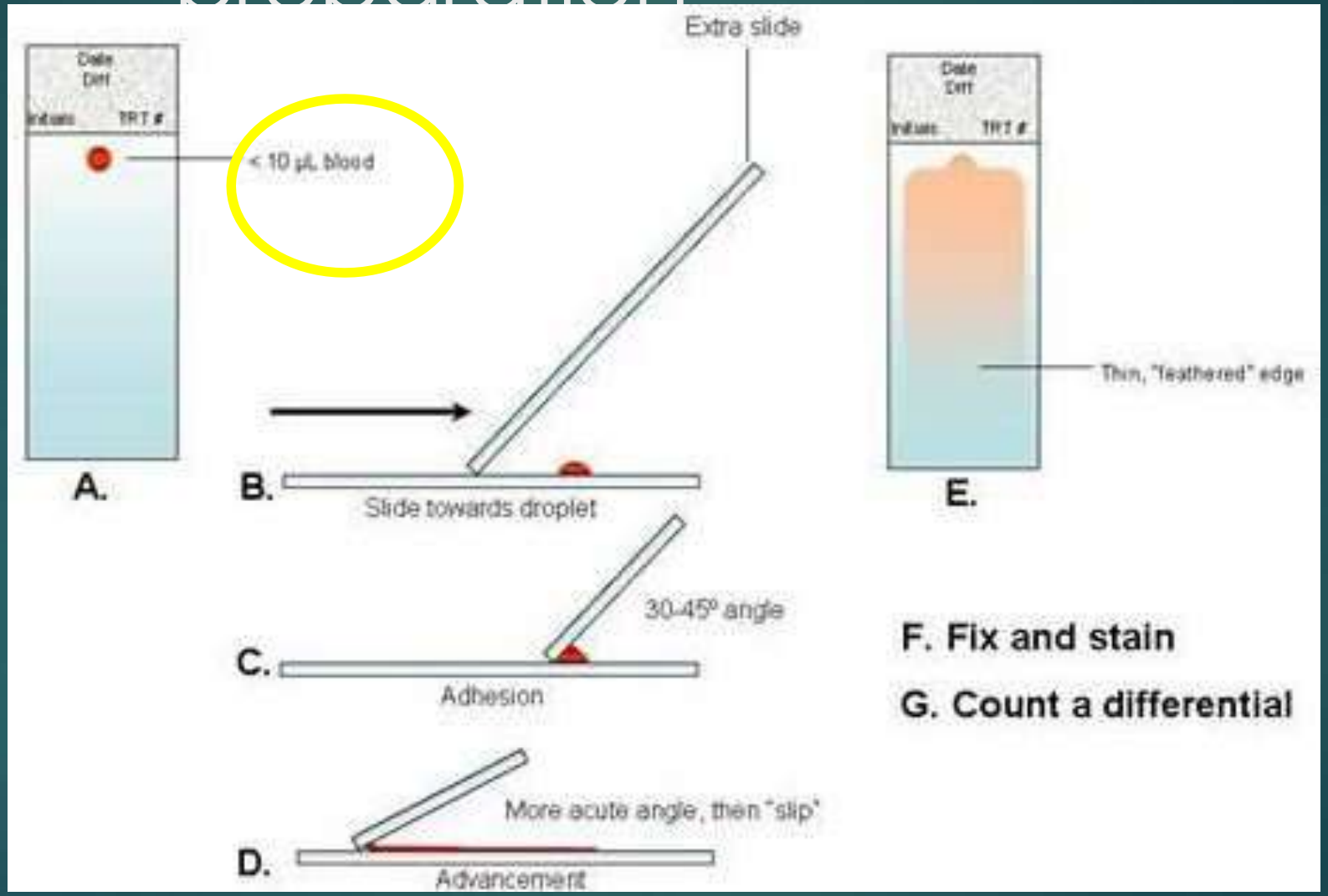
Precaution

- The blood film should be done immediately from fresh blood or within an hour of sampling in case of anticoagulated blood.

Place a small drop of well mixed blood near one end of the slide with applicator stick

Slides – 75x25mm, 1mm thick

Methods of preparation



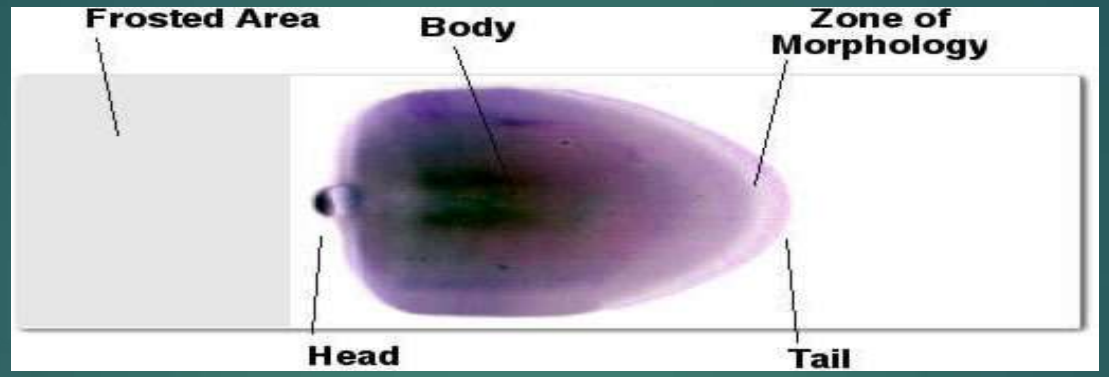
Quality of the smear

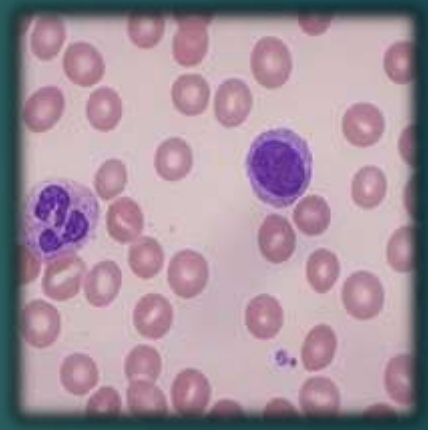
- Size of the blood drop
- Angle applied to the spreader
- Pressure , speed and steadiness in pushing the spreader

Precautions

- Air drying for 15- 20 minutes at-least.
- Slow drying should be avoided**
- Avoid humidity (no breath blowing)

Well made smear





Fixing of the blood film

- Fixing is done to preserve the morphology of the cells
- This should be done without delay
- Films should not be left unfixed for more than a few hours
- Acetone free methanol is used most commonly
- Ethanol can also be used
- Water contamination should be avoided

Romanowsky stains

- Used universally for routine staining of blood films.
- The remarkable property of the Romanowsky dyes of making subtle distinctions in shades of staining, and of staining granules differentially.
- All routine stain based on Romanowsky principle MUST contain two components:

1. A cationic or basic dye (methylene blue or its oxidation products such as azure B), which binds to anionic sites and gives a blue-grey color to nucleic acids (DNA or RNA), nucleoproteins, granules of basophils and weakly to granules of neutrophils
2. An anionic or acidic dye such as eosin Y or eosin B, which binds to cationic sites on proteins and gives an orange-red color to hemoglobin and eosinophil granules.

Types

- Leishman Stain
- Giemsa Stain
- Wright Stain
- Jenner
- May–Grünwald–Giemsa
- Wright–Giemsa
- Jenner–Giemsa
- Field Stain
- azure B– eosin Y methods

- Jenner is the simplest and Giemsa is the most complex.
- Leishman stain occupies an intermediate position, is still widely used in the routine staining of blood films, although the results are inferior to those obtained by the combined May–Grünwald–Giemsa, Jenner–Giemsa, and azure B– eosin Y methods

- A pH to the alkaline side of neutrality accentuates the azure component at the expense of the eosin and vice versa.
- A pH of 6.8 is usually recommended for general use.
- When looking for malaria parasites, a pH of 7.2 is recommended to see Schüffner dots.

Leishman stain

AIR DRY SMEAR & PLACE THE SMEAR ON STAINING BAR

↓

COVER THE SMEAR WITH **8-10 DROPS** LEISHMAN'S STAIN FOR PS **2 MIN** AND FOR BONE MARROW **8-10 MIN.**

↓

ADD D/W **DOUBLE THE DROPS OF STAIN** ON THE SLIDE FOR PS **5-7 MIN** AND FOR BONE MARROW **15 MIN.**

↓

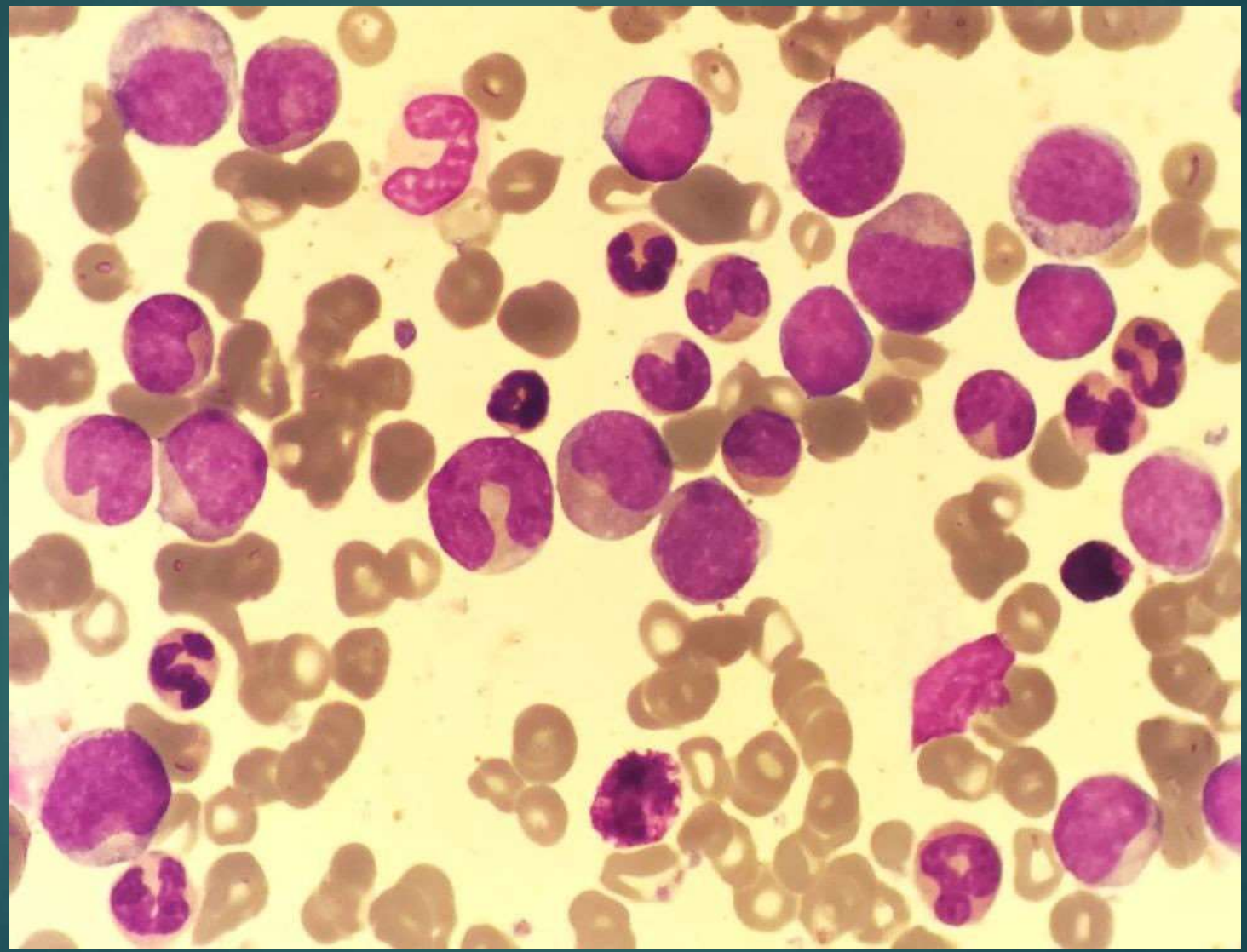
MIX BY BLOWING OR USING A DROPPER

↓

WASH THE SLIDE UNDER RUNNING TAP WATER & BACK OF THE SLIDE HAS BEEN WIPED CLEAN

↓

SET IT UPRIGHT TO AIR DRY

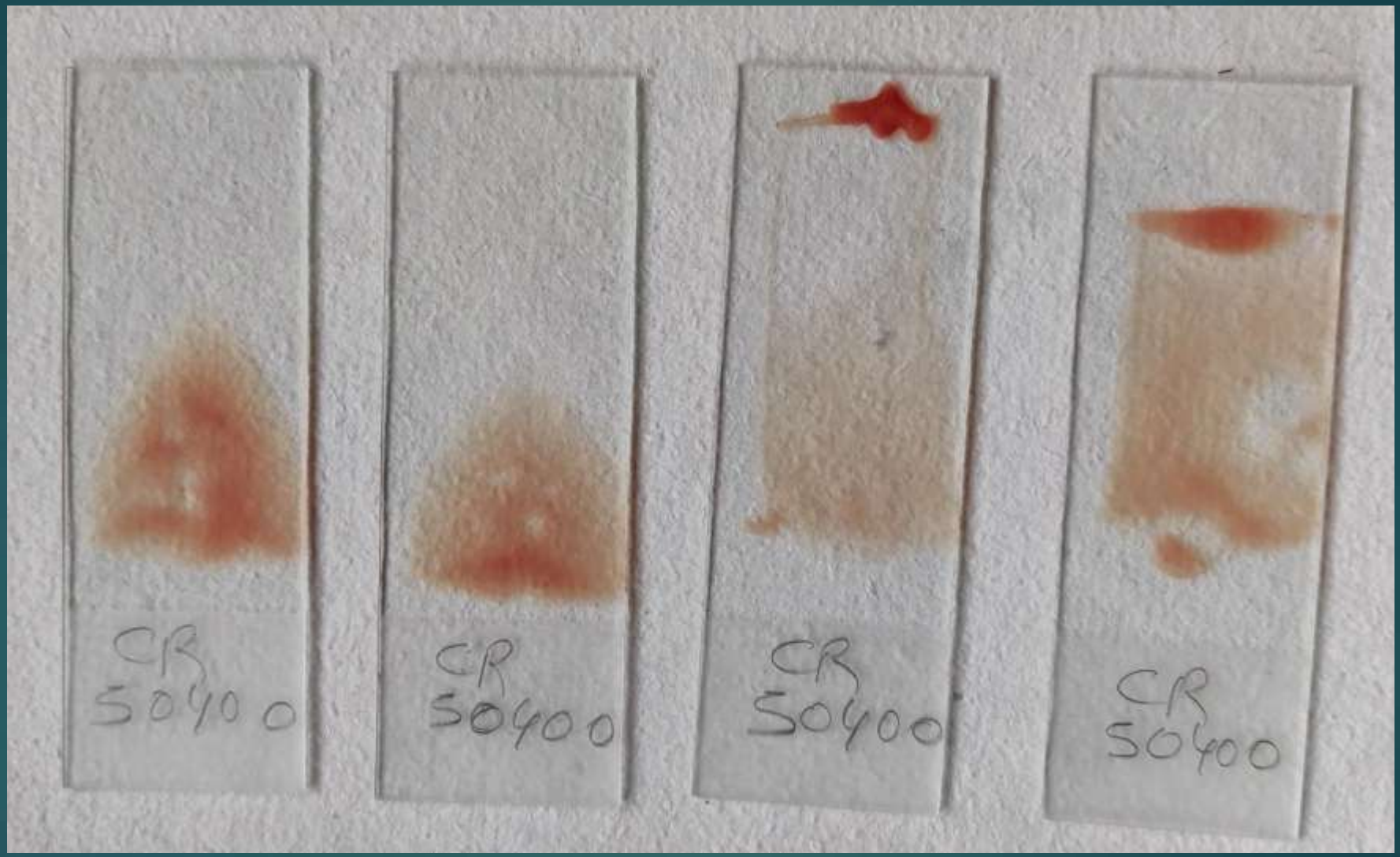


COLOUR RESPONSES OF BLOOD CELLS TO ROMANOWSKY STAINING

Cellular Component	Colour
Nuclei	
Chromatin	Purple
Nucleoli	Light blue
Cytoplasm	
Erythroblast	Dark blue
Erythrocyte	Dark pink
Reticulocyte	Grey-blue
Lymphocyte	Blue
Metamyelocyte	Pink
Monocyte	Grey-blue
Myelocyte	Pink
Neutrophil	Pink/orange
Promyelocyte	Blue
Basophil	Blue
Granules	
Promyelocyte (primary granules)	Red or purple
Basophil	Purple-black
Eosinophil	Red-orange
Neutrophil	Purple
Toxic granules	Dark purple
Platelet	Purple
Other Inclusions	
Auer body	Purple
Cabot ring	Purple
Howell–Jolly body	Purple
Döhle body	Light blue

Problems encountered

- Faulty smear preparation
- Faulty technique
- Storage artifacts
- Staining artifacts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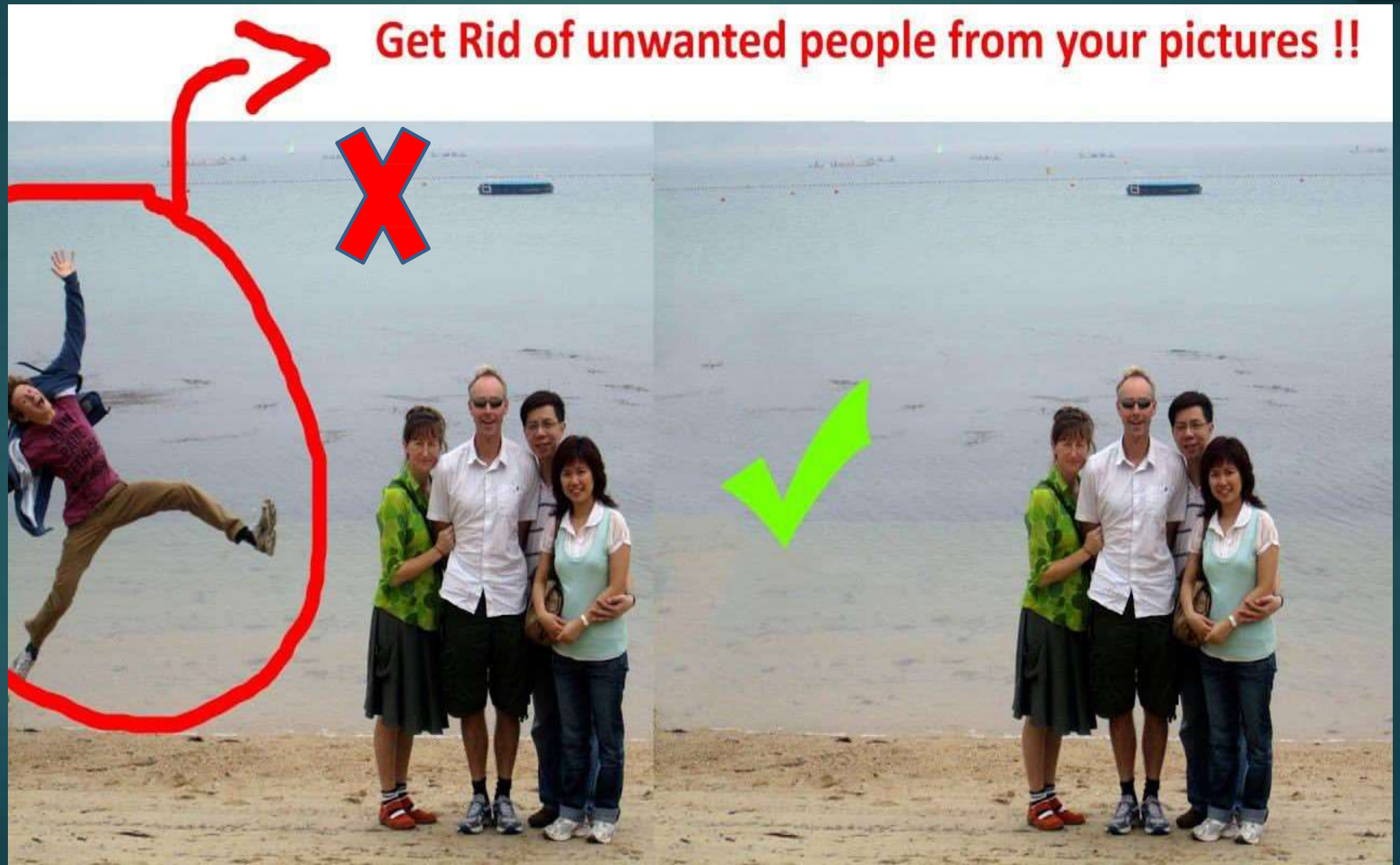
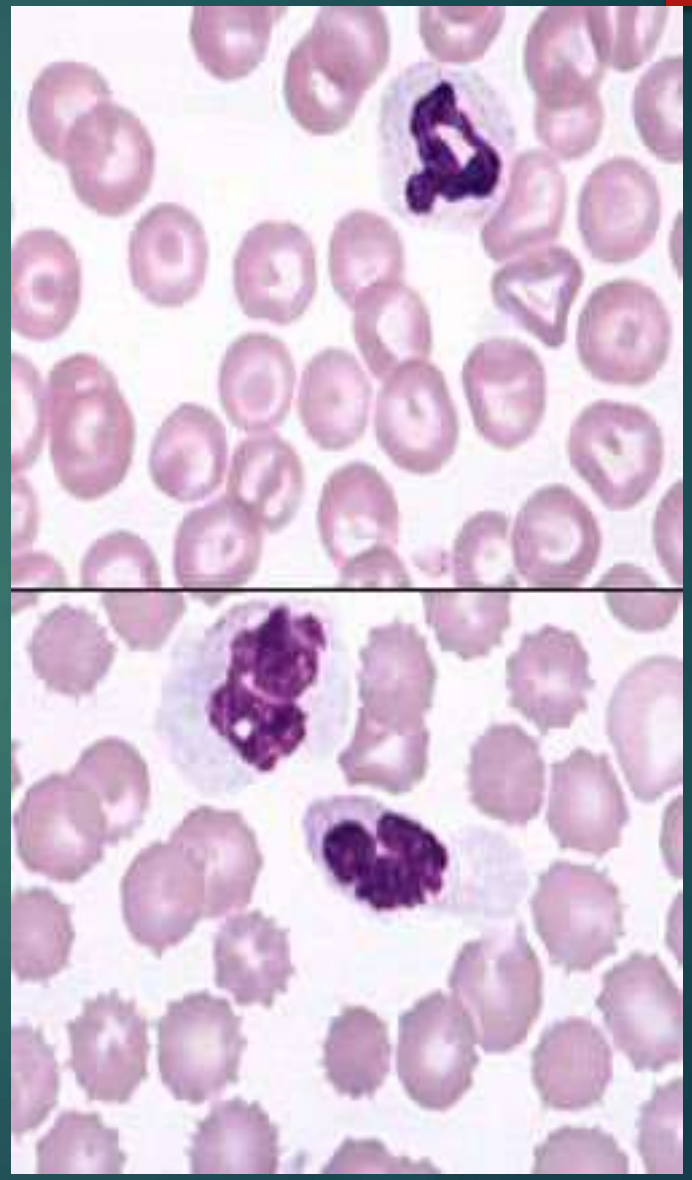
Artifacts



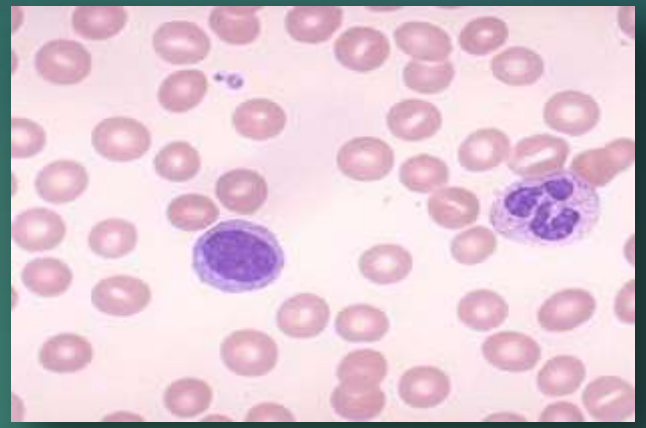
Storage (EDTA) artifacts

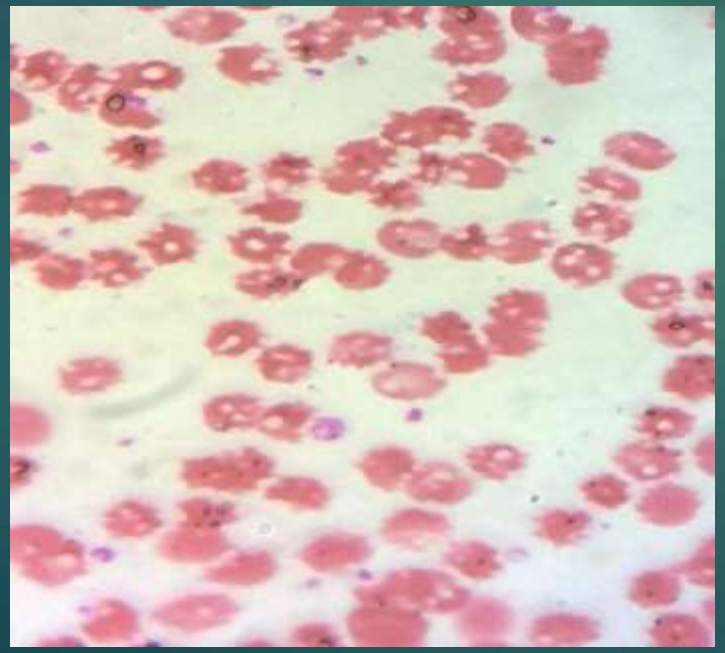
- Cytoplasmic vacuolation in neutrophils
- Loss of central pallor in RBCs can cause misidentification of normal erythrocytes as spherocytes
- Crenated RBCs



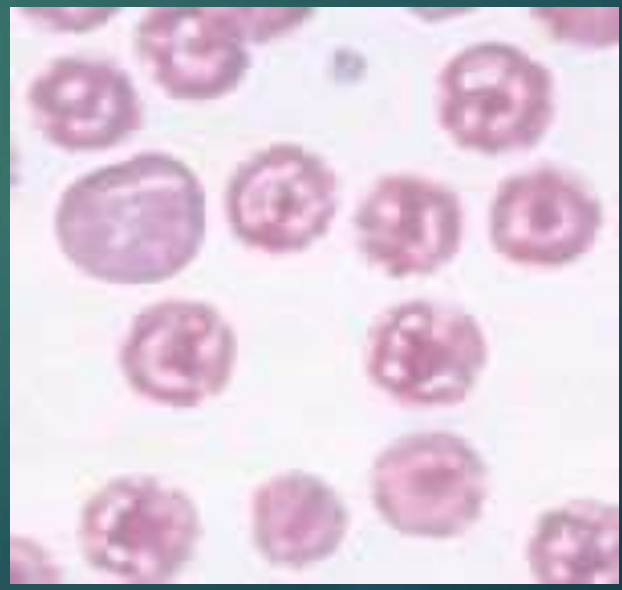
Excessive water on the slide or in the stain producing refractile edges on erythrocytes

Normal Smear

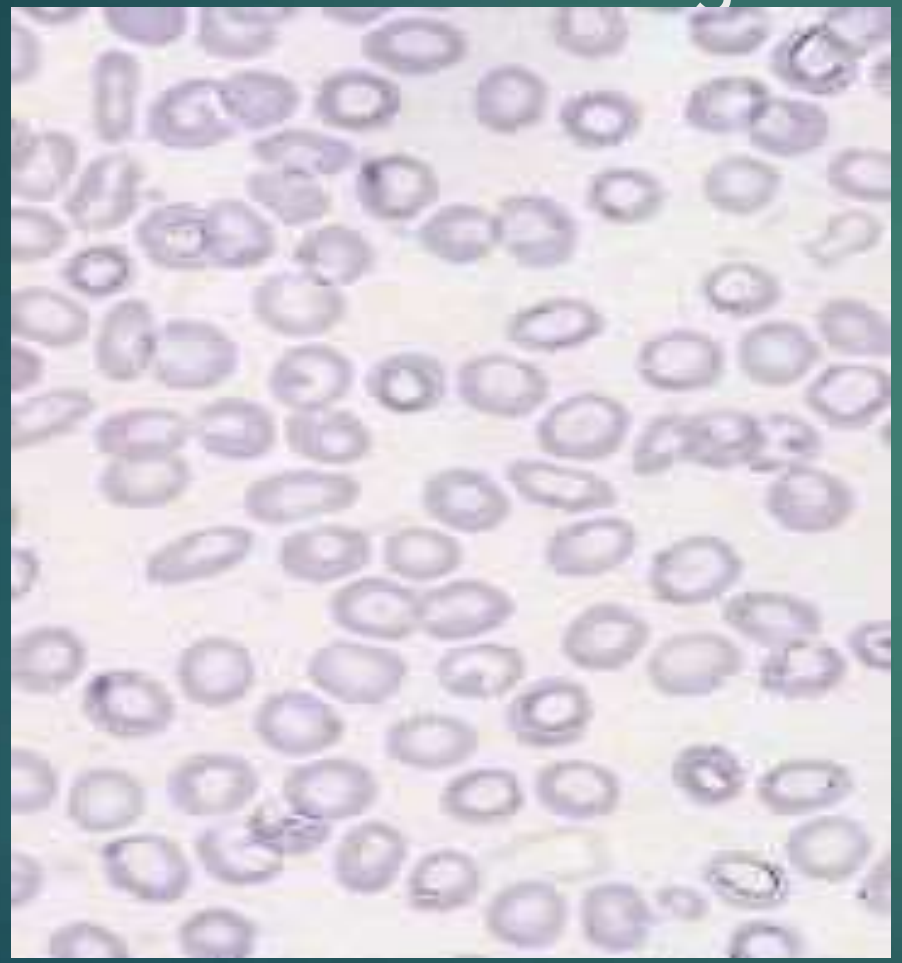




Water Artifact



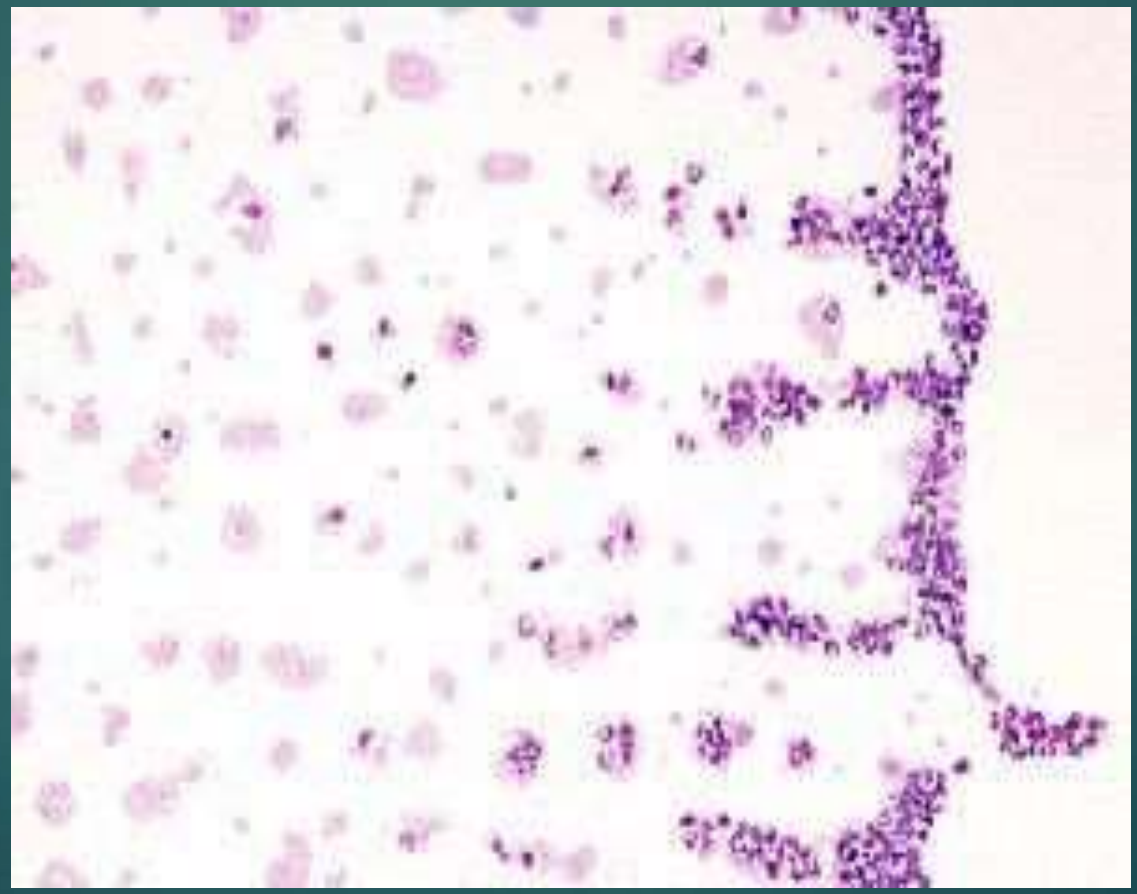
Artefacts



- **Fixation artefact**
- Occurs when there is water in the methanol used for fixation of the blood film.
- This leads to refractile rings in red cells and makes it quite impossible to assess red cell morphology.

Tailing artefact

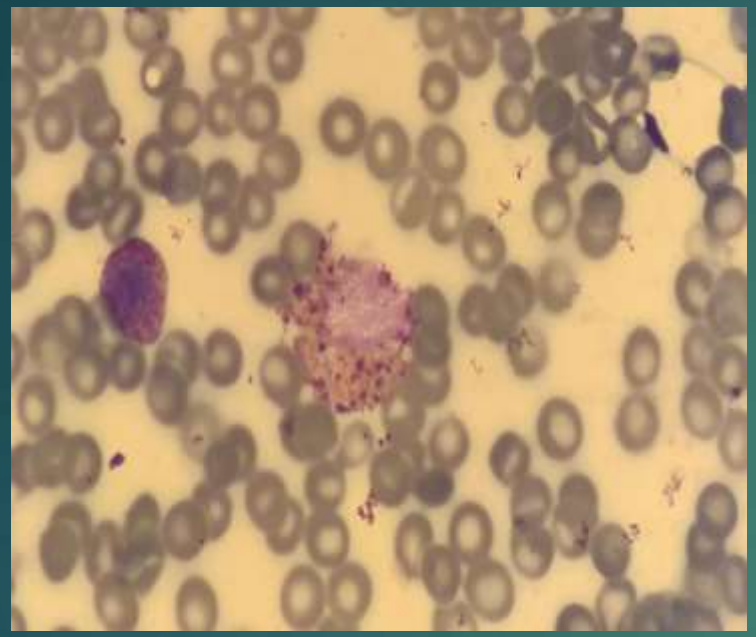
Poor distribution of leukocytes

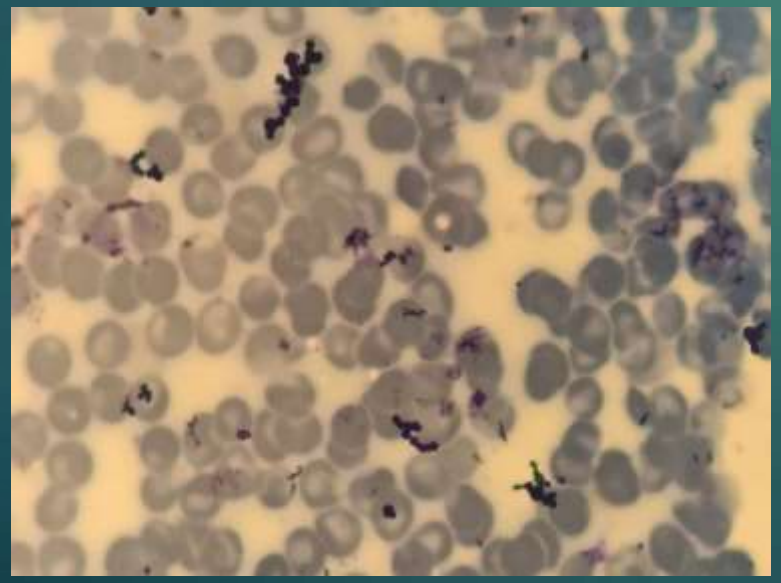


STAINING ARTIFACTS

- Caused by an incorrect **pH of buffer**
- Delayed fixation of blood smear
- Smear from heparin anticoagulated blood
- Poor batch of staining reagents
- Stain deposition due to evaporation of methanol

Stains Artifacts – Stain Deposition

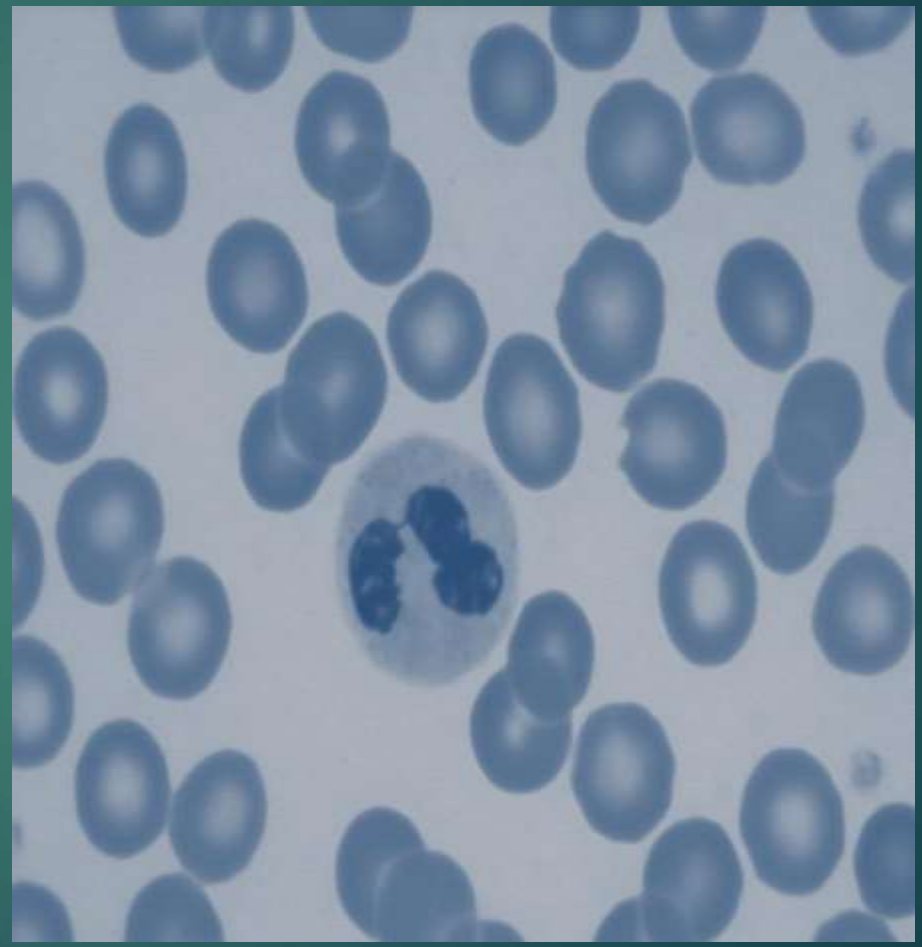




- Aged staining solutions
- Inadequate rinsing of slides
- no filteration
- Finer precipitate can effectively mimic epicellular parasites or bacteria
- Dust

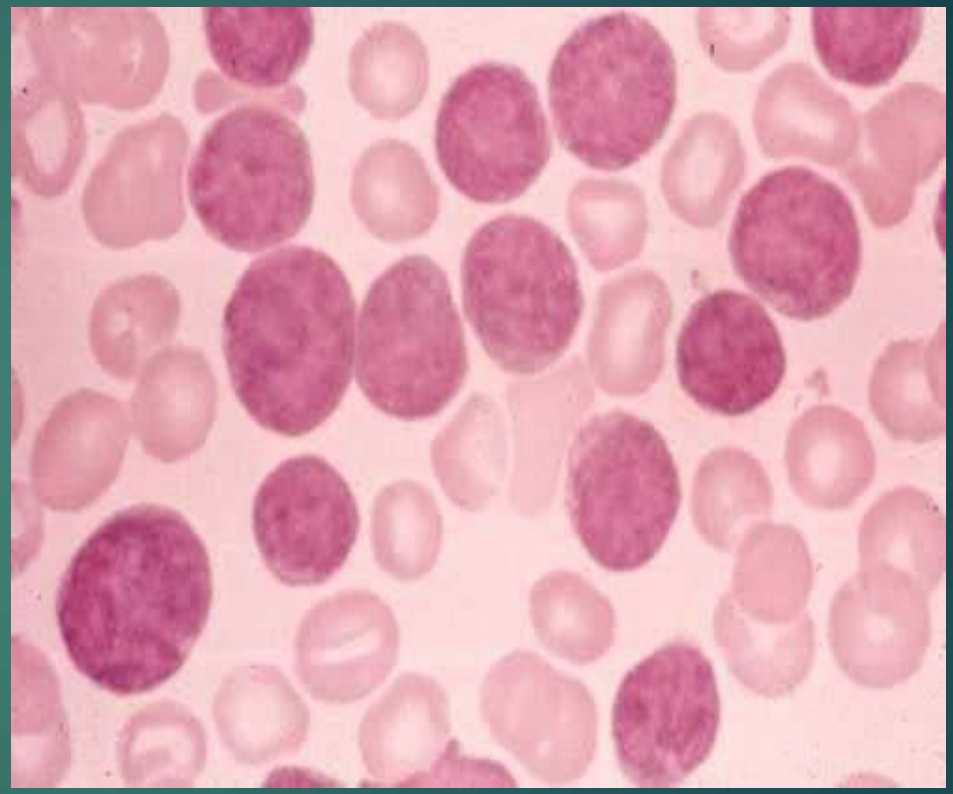
Too blue appearance

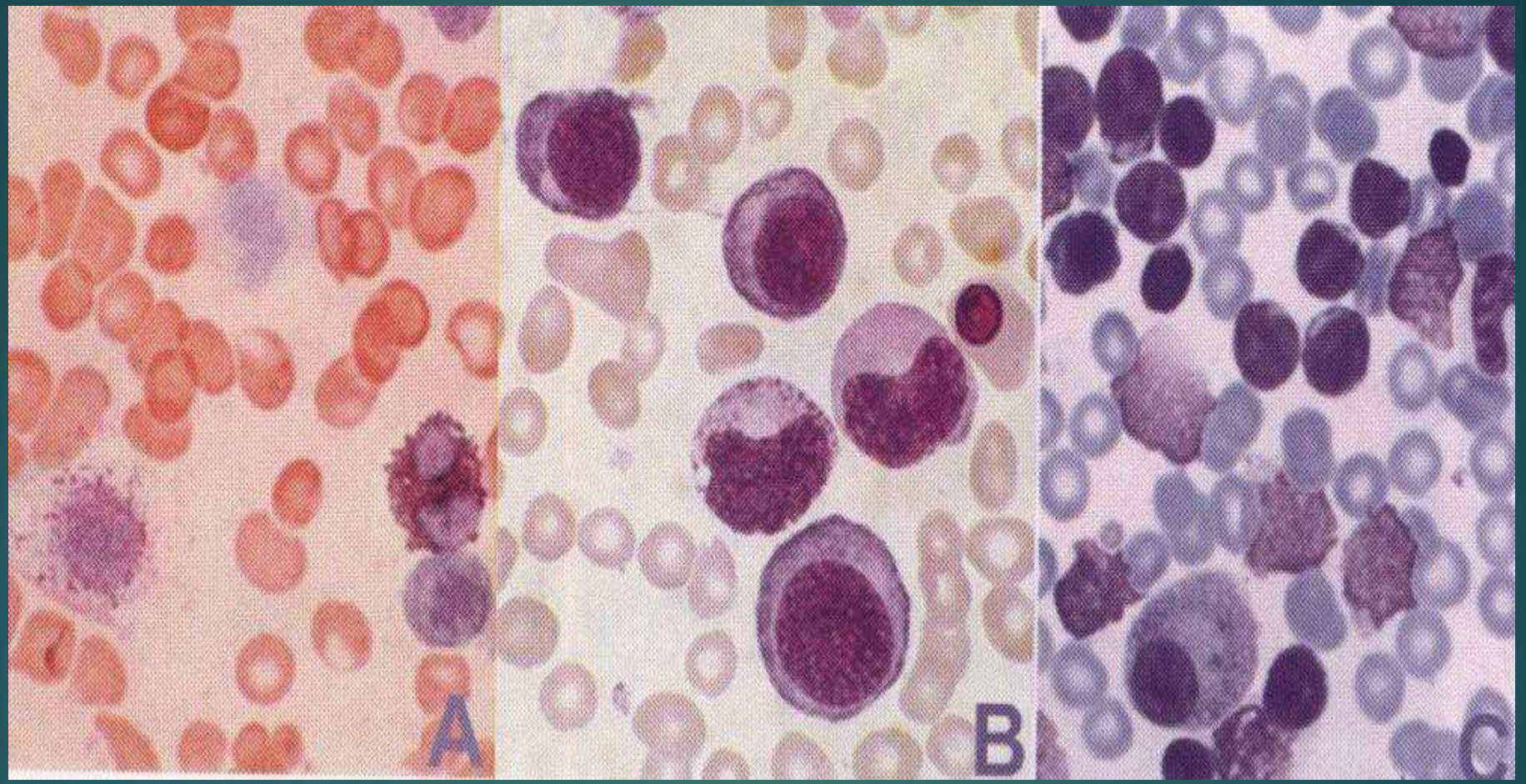
- Eosin concentration too low
- Staining time too short
- Inadequate time in buffer solution
- Heparinized blood



Too Pink appearance

- Incorrect proportion of Azure B: Eosin Y
- Buffer pH too low
- Excessive washing in buffer solution





TOO ACIDIC SUITABLE TOO BASIC

leucocyte cytochemistry

- Myeloperoxidase
- Sudan black B
- Neutrophil alkaline phosphatase acid phosphatase
- Periodic acid-Schiff reaction
- Esterases
- Toluidine blue stain

Cytochemical Stains in Common Use

Myeloperoxidase (MPO)

- Located in the primary and secondary granules of granulocytes and their precursors, in eosinophil granules and in the azurophil granules of monocytes
- The MPO in eosinophil granules is cyanide resistant, whereas that in neutrophils and monocytes in cyanide sensitive

Principle of Test

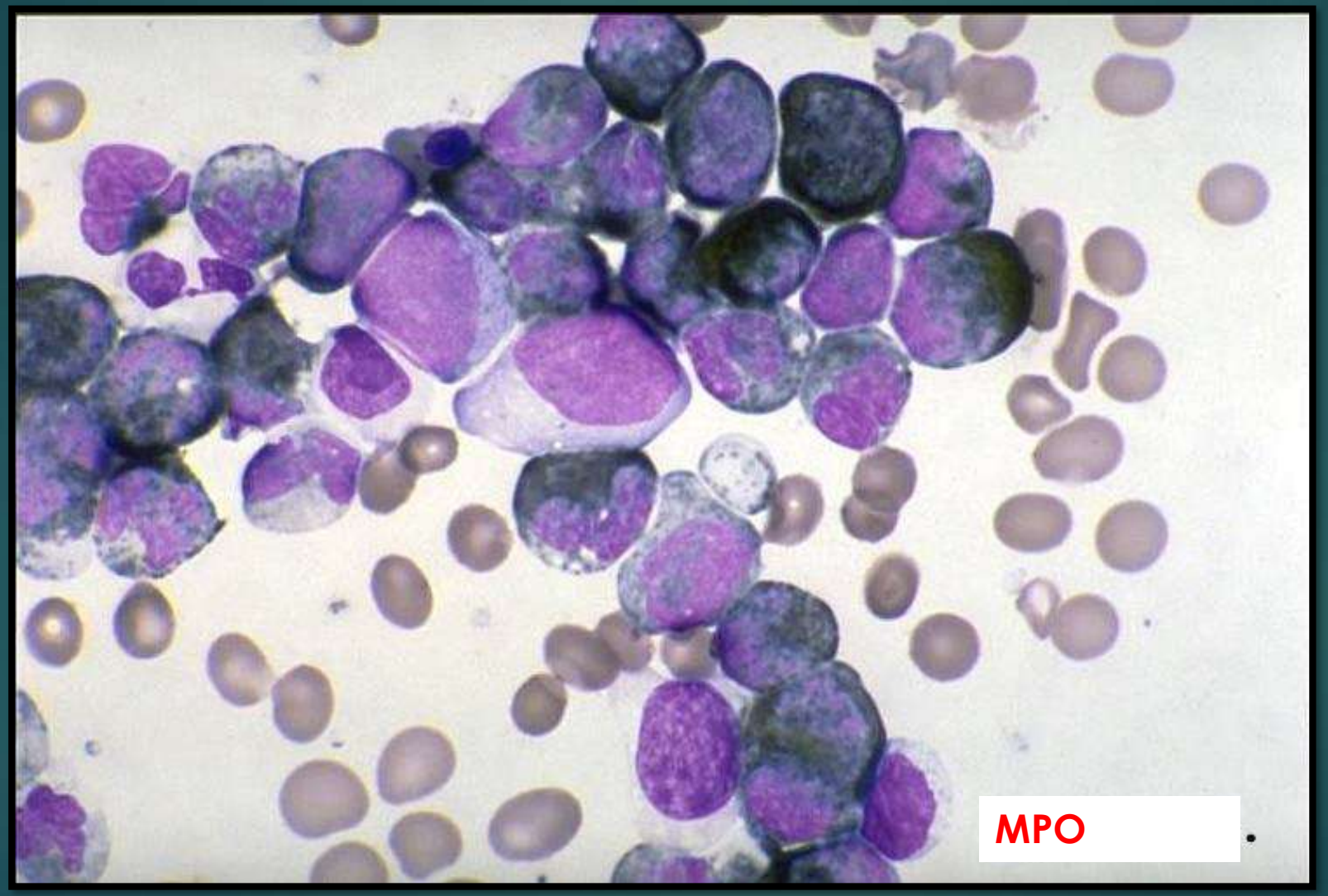
- MPO splits H_2O_2, and in the presence of a chromogenic electron donor, forms an insoluble reaction product
- 3,3'-diaminobenzidine (DAB) is the preferred chromogen
- The reaction product is stable, insoluble and non-diffusible

Interpretation of the result

- The reaction product is brown and granular
- Red cells and erythyroid precursors show diffuse brown cytoplasmic staining
- The most primitive myeloblasts are negative, with granularly positively appearing progressively as they mature towards the promyelocyte stage
- Promyelocytes and myelocytes are the most strongly staining cells in the granulocyte series

- Metamyelocytes and neutrophils have progressively fewer positive (secondary) granules
- Eosinophil granules stain strongly, and the large specific eosinophil granules are easily distinguished from neutrophil granules
- Monoblasts and monocytes may be negative or weak positive
- MPO activity is present in basophil granules but is not demonstrable in mature basophils by the DAB reaction

Cytochemical Techniques in Common Use



Cytochemical Techniques in Common Use cont'd

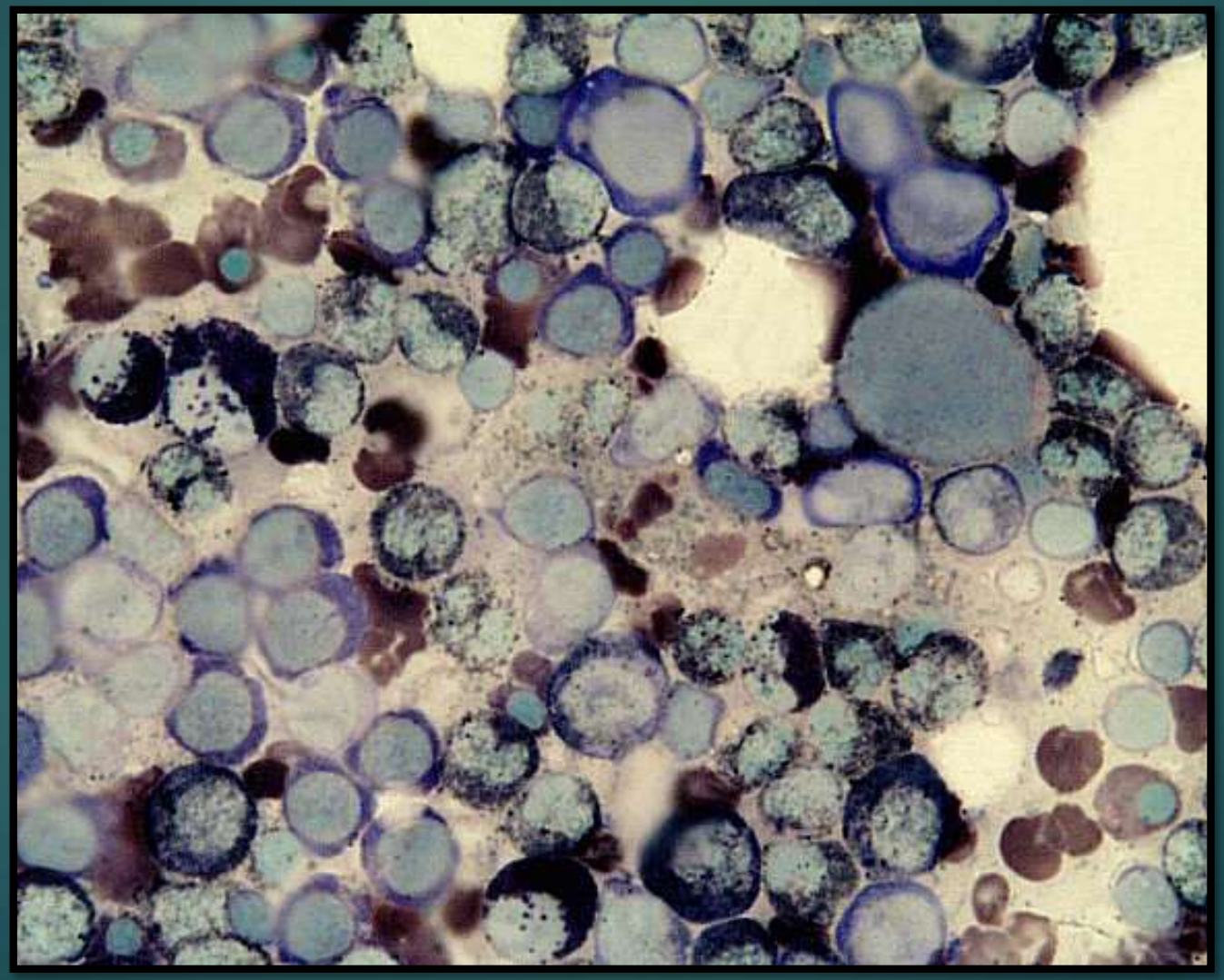
Sudan Black B

- A lipophilic dye that binds irreversibly to an undefined granule component in granulocytes, eosinophils and some monocytes
- It cannot be extracted from the stained granules by organic dye solvents
- Gives comparable information to that of MPO staining

Interpretation of the result

- The reaction product is black and granular
- The results are essentially similar to those seen with MPO staining, both in normal and leukemic cells

Cytochemical Techniques in Common Use cont'd



Cytochemical Techniques in Common Use cont'd

Esterases

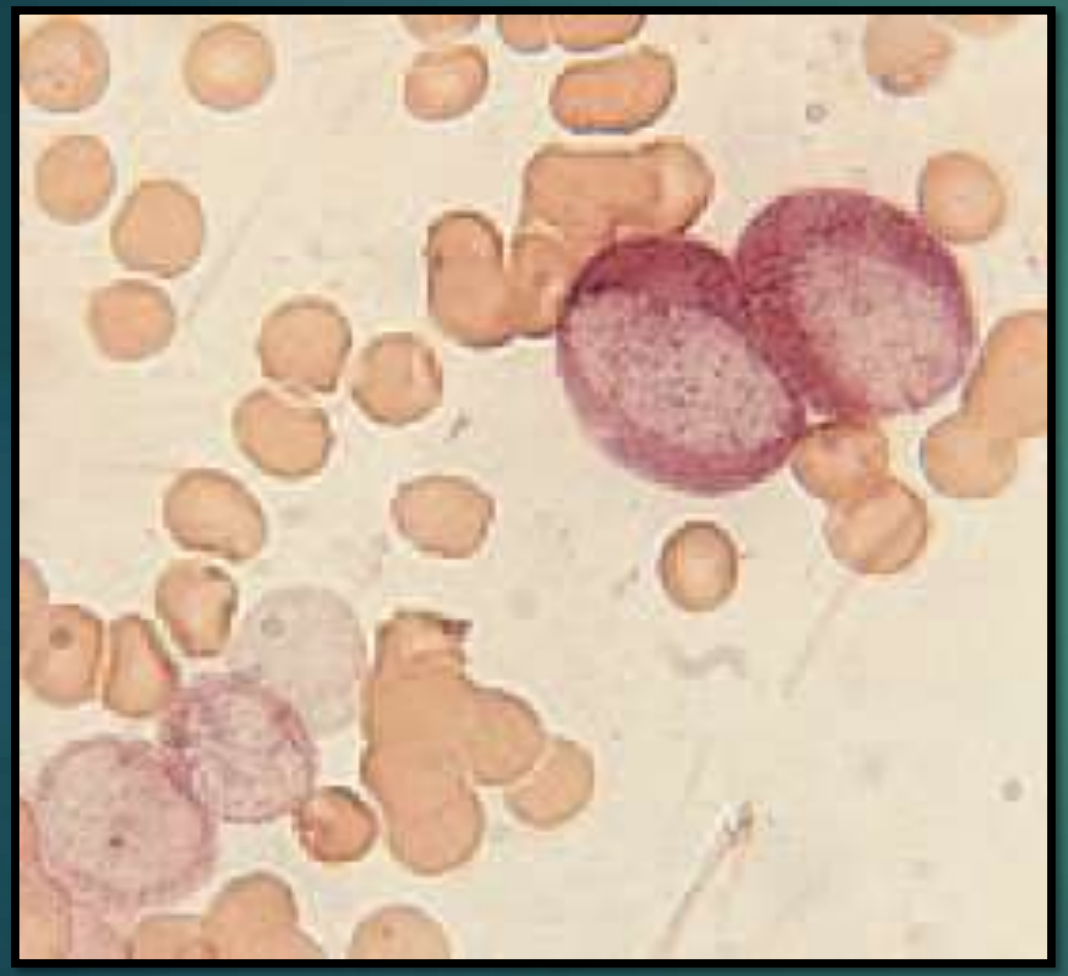
- **"Specific" esterase** of granulocytes, stains specifically with naphthol AS-D chloroacetate esterase (chloroacetate esterase, CAE)
- **"Non-specific"** esterase (NSE), stains with α-naphthyl acetate esterase (ANAE) and α-naphthyl butyrate esterase (butyrate esterase, BE)

Cytochemical Techniques in Common Use cont'd

Interpretation of the result with AS-D chloroacetate esterase

- The reaction product is bright red
- It is confined to cells of the granulocyte series and mast cells
- Cytoplasmic CAE activity appears as myeloblasts mature to promyelocytes
- Positivity in myeloblasts is rare, but promyelocytes and myelocytes stain strongly, with reaction product filling the cytoplasm
- More mature granulocytes stain strongly but less intensely

Cytochemical Techniques in Common Use cont'd



Leukaemic blast cells stained for chloroacetate
esterase (CAE) activity

Cytochemical Techniques in Common Use cont'd

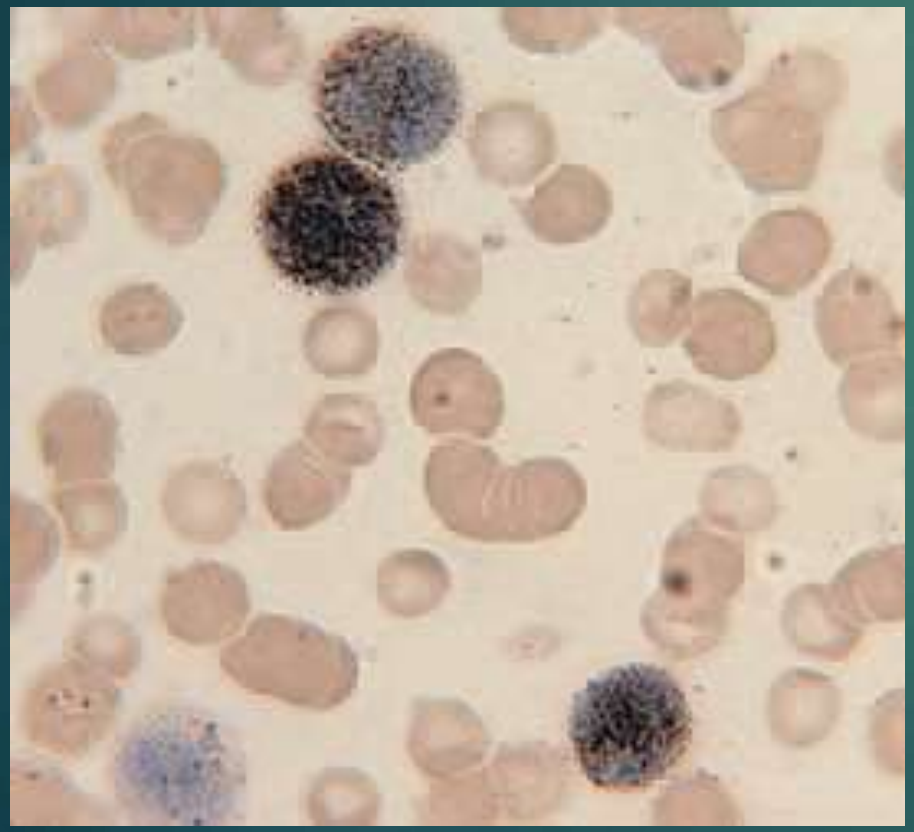
Interpretation of the result with α-Naphthyl butyrate esterase

- The reaction product is brown and granular
- The majority of monocytes (>80%) stain strongly, the remainder showing some weak staining
- Neutrophils, eosinophils, basophils and platelets are negative
- B lymphocytes are negative and T lymphocytes are unreliably stained
- In the bone marrow, monocytes, their precursors and macrophages stain strongly. α-naphthyl butyrate is more specific for identifying a monocytic component in AML than α-naphtyl acetate

Interpretation of result with α-naphthyl Acetate Esterase

- The reaction product is diffuse red/brown in color
- Normal and leukemic monocytes stain strongly
- Normal granulocytes are negative, but in myelodysplasia or AML may give positive reactions of varying intensity

Cytochemical Techniques in Common Use cont'd



Leukaemic blast cells stained for a-naphthyl
acetate esterase (ANAE) activity

Cytochemical Techniques in Common Use cont'd

Neutrophil Alkaline Phosphatase (NAP)

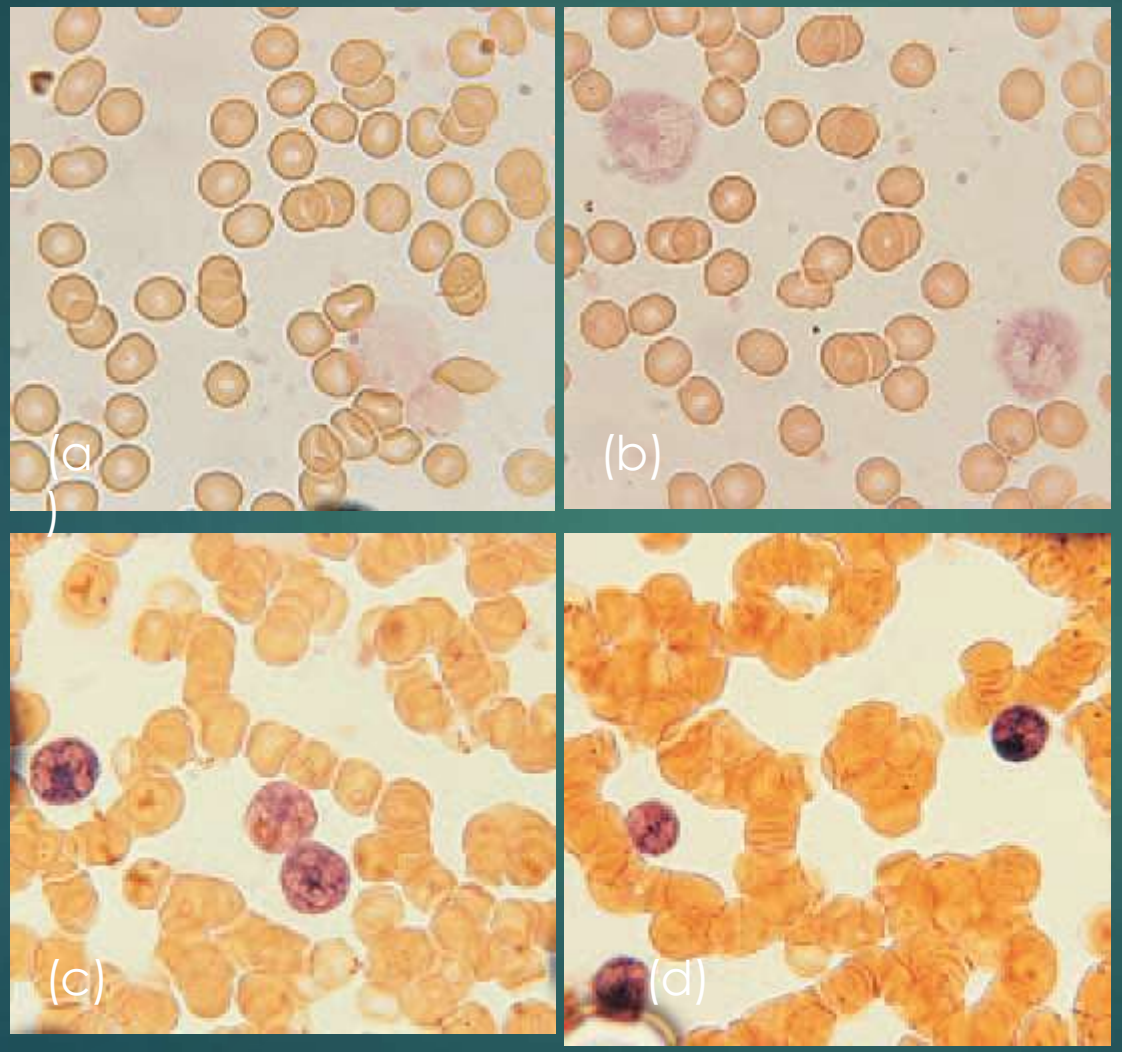
- Alkaline phosphatase activity is found predominately in mature neutrophils, with some activity in metamyelocytes
- Although demonstrated as a granular reaction product in the cytoplasm, enzyme activity is associated with a poorly characterized intra-cytoplasmic membranous component distinct from primary or secondary granules

Cytochemical Techniques in Common Use cont'd

Interpretation of the result

- The reaction product is blue and granular
- The intensity of reaction product in neutrophils varies from negative to strongly positive, with coarse granules filling the cytoplasm and overlying the nucleus
- An overall score is obtained by assessing the stain intensity in 100 consecutive neutrophils, with each neutrophil scored on a scale of 1-4 as follows:

Cytochemical Techniques in Common Use cont'd



Neutrophil alkaline phosphatase (NAP) reaction (method of Ackerman) showing cells with reactions graded 0–4:

(a) neutrophil with a score of 0 plus a lymphocyte which is also negative

(b) two band cells with a score of 1

(c) two neutrophils with a score of 2 and one with a score of 3

(d) two neutrophils with a score of 4 and one with a score of 2

Cytochemical Techniques in Common Use cont'd

- In normal individuals, it is rare to find neutrophils with scores of 3, and scores of 4 should not be present
- There is some physiological variation in NAP scores:
  - Newborn babies, children and pregnant women have high scores
  - Premenopausal women have, on average, scores one-third higher than men
- In pathological states, the most significant diagnostic use of the NAP score is in chronic myeloid leukemia
  - In the chronic phase of the disease, the score is almost invariably low usually zero
  - Transient rises may occur with intercurrent infection

Cytochemical Techniques in Common Use cont'd

- In myeloid blast transformation or accelerated phase, the score rises
- Low scores are also commonly found in paroxysmal nocturnal hemoglobinuria (PNH) and the very rare condition of hereditary hypophosphatasia
- There are many causes of a raised NAP score, notably in:
  - Neutrophilia of infection
  - Polycythaemia rubra vera (PRV)
  - Leukemoid reactions
  - Hodgkin's disease
- In aplastic anemia, the NAP score is high, but falls if PNH supervenes

Cytochemical Techniques in Common Use cont'd

Acid Phosphatase Reaction

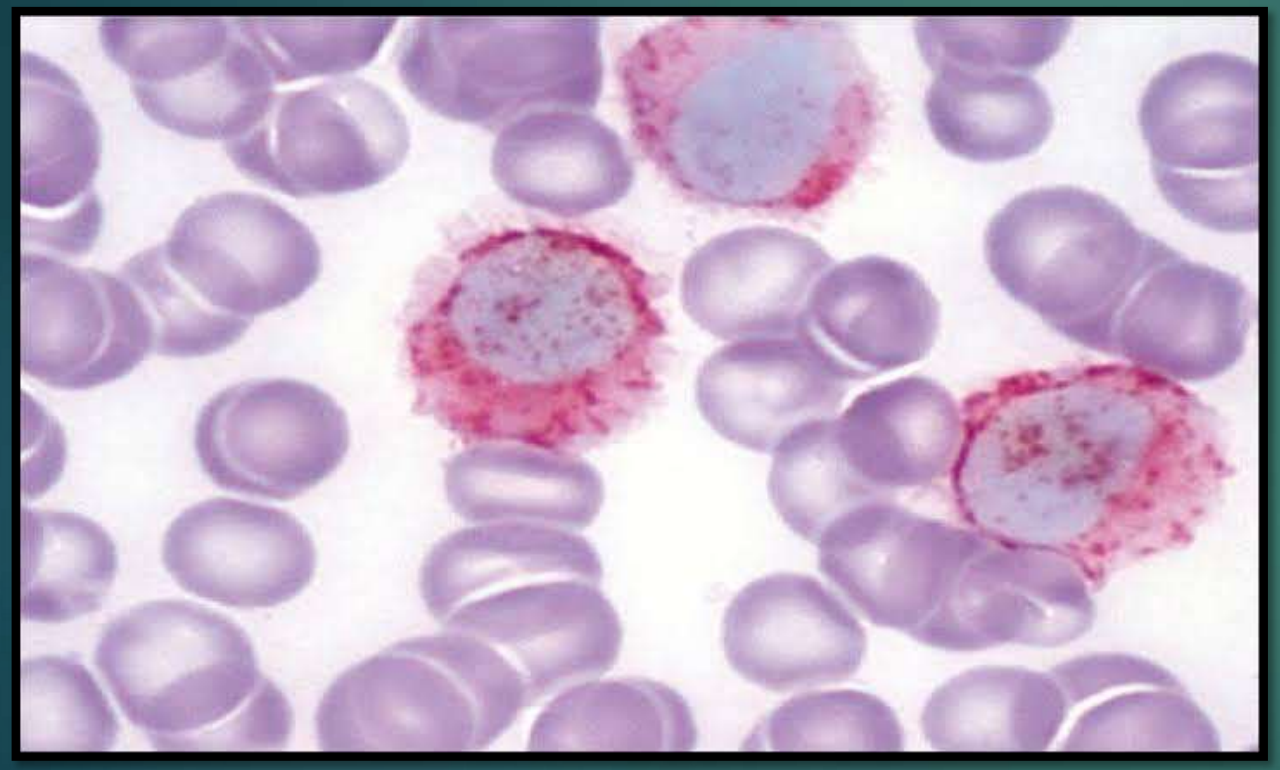
- Its main diagnostic use is in the diagnosis of T-cell acute leukemias and hairy cell leukemia
- The pararosaniline method is recommended for demonstrating positivity in T lymphoid cells

Cytochemical Techniques in Common Use cont'd

Interpretation of the result

- The reaction product is red with a mixture of granular and diffuse positivity
- In T cells, acid phosphates are an early differentiation feature
- Almost all acute T-cell leukemias show strong activity
- In T-cell acute leukemias, the activity is usually highly localized (polar)
- Granulocytes are strongly positive
- Monocytes, eosinophils and platelets show variable positivity
- In the bone marrow, macrophages, plasma cells and megakaryocytes are strongly positive

Cytochemical Techniques in Common Use cont'd

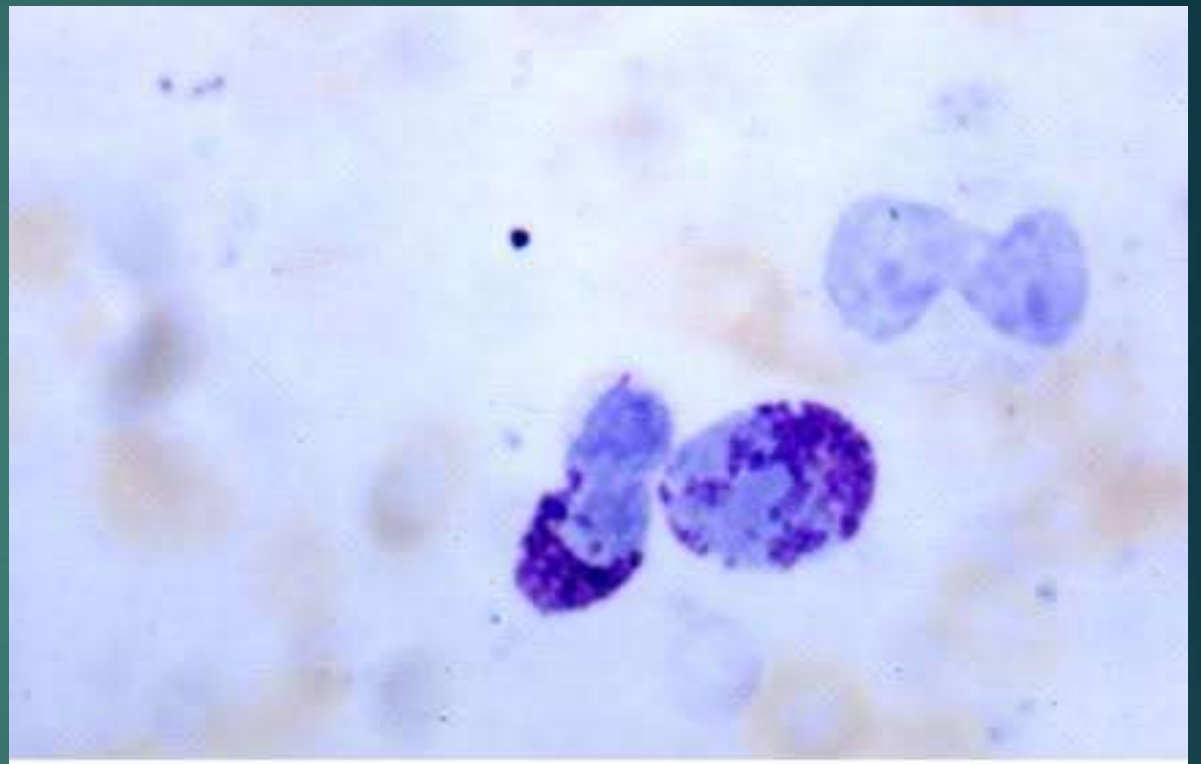


Tartarate resistant Acid phosphatase stain in hairy cell leukaemia

Cytochemical Techniques in Common Use cont'd

Toluidine Blue Stain

- Toluidine blue staining is useful for the enumeration of basophils and mast cells
- It binds strongly to the granules in these cells, and is particularly useful in pathological states where the cells may not be easily identifiable on Romanowsky stains

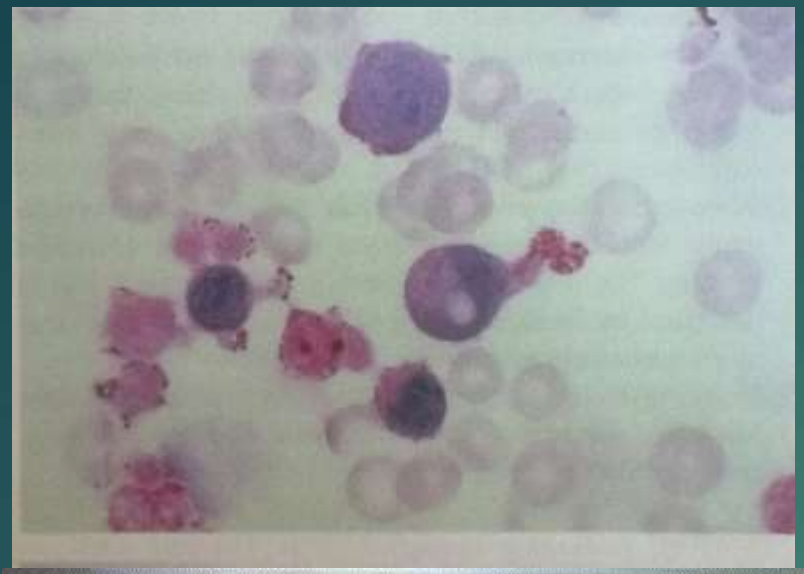


Periodic Acid – Schiff [PAS] Reaction

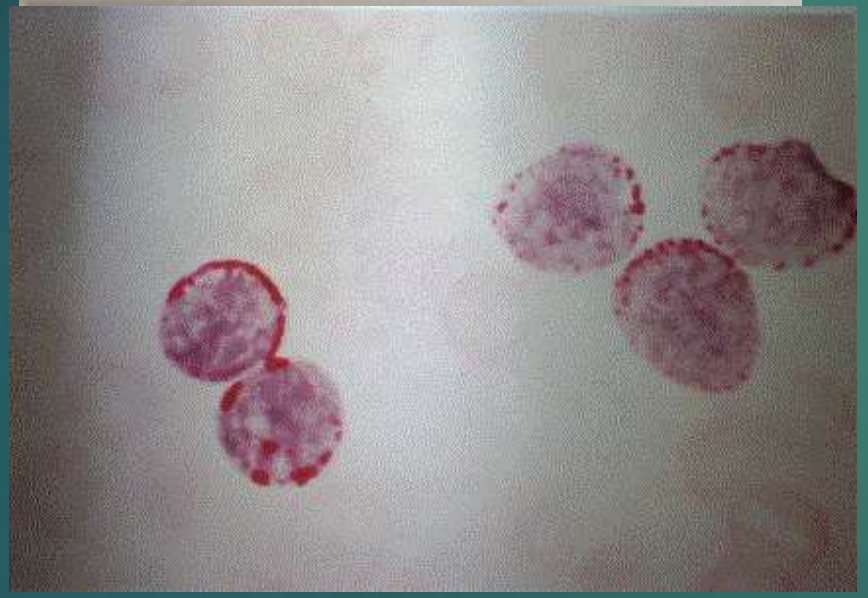
- **Principle**: the stain indicates the presence of muccoproteins , glycoproteins and high molecular weight carbohydrates in blood cells.
- The reaction product is red, with intensity ranging from pink to bright red.
- Granulocyte precursors show diffuse weak positivity, with neutrophils showing intense confluent granular positivity.
- Monocytes and their precursors show variable diffuse positivity with superimposed fine granules, often at the periphery of the cytoplasm.
- Normal erythroid precursors and red cells are negative.
- Megakaryocytes and platelets show variable, usually intense, diffuse positivity.

- Lymphoblasts show variable PAS-positive cytoplasmic granules or blocks on a clear background; it is block positivity on a clear background that is most characteristic of lymphoblasts rather than myeloblasts.
- When immunophenotyping is available, the PAS reaction is redundant for the diagnosis of ALL.

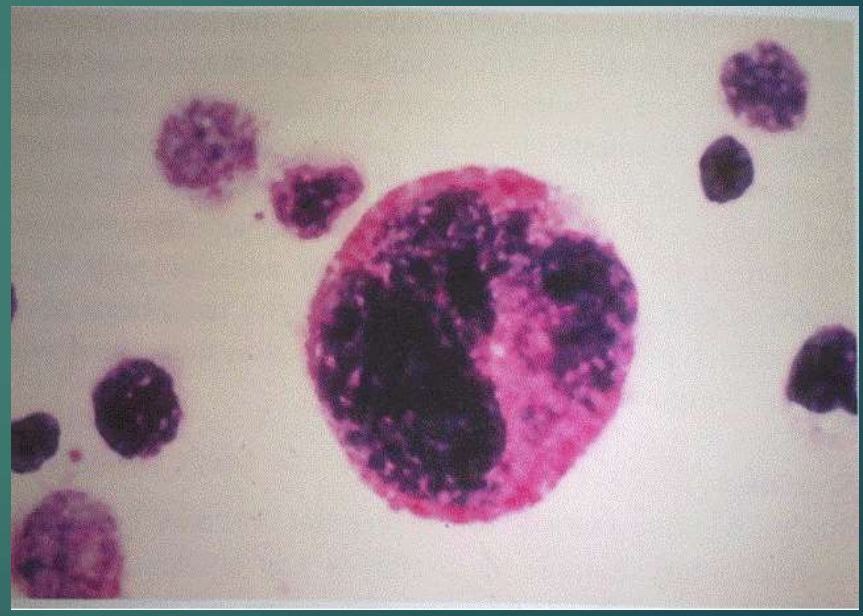
- It can still be useful in AML and MDS to identify abnormal erythroblasts and dysplastic megakaryocytes



Positive PAS stain acute megakaryocytic leukemia AML, M7.



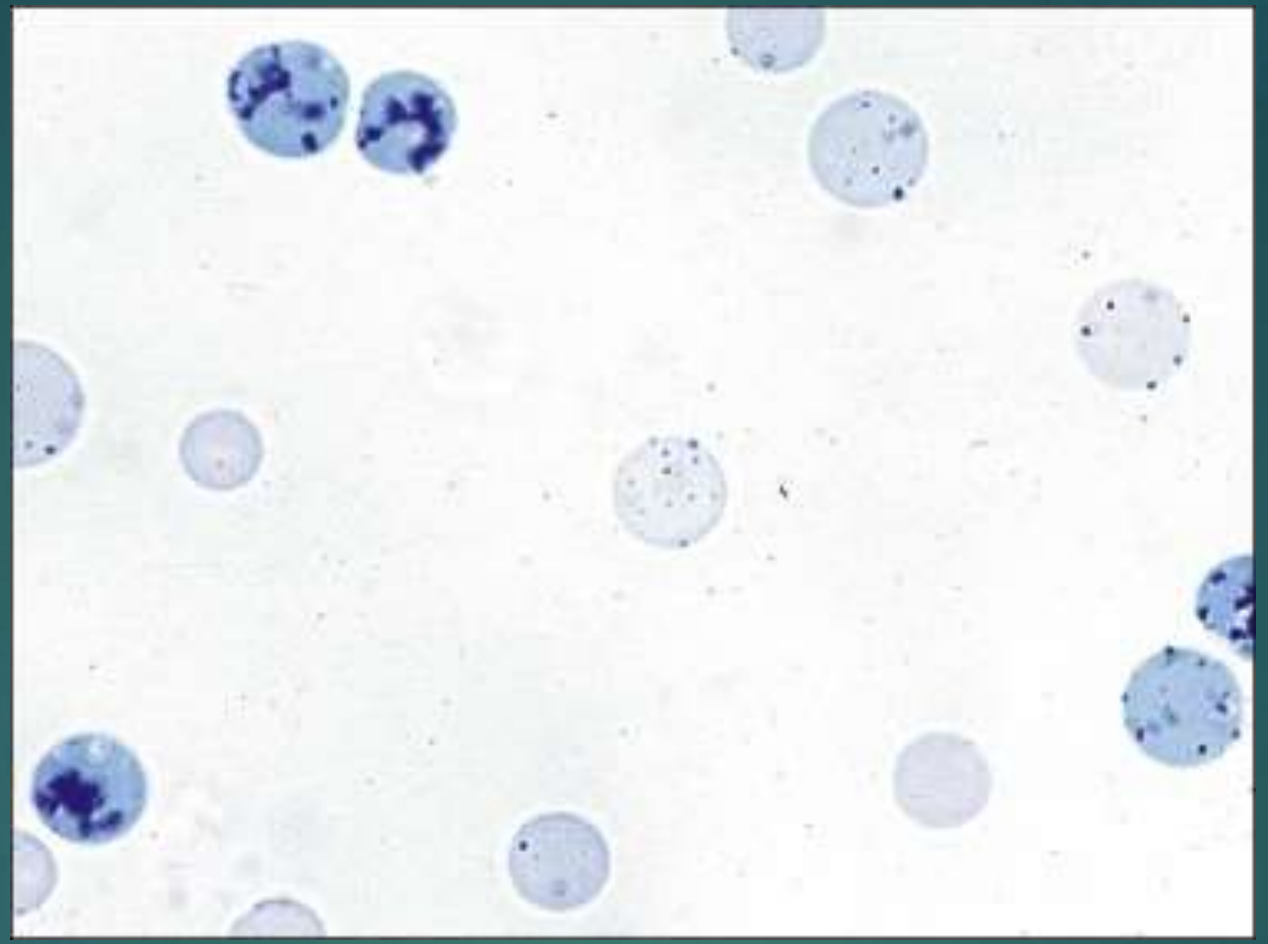
Positive PAS stain in ALL



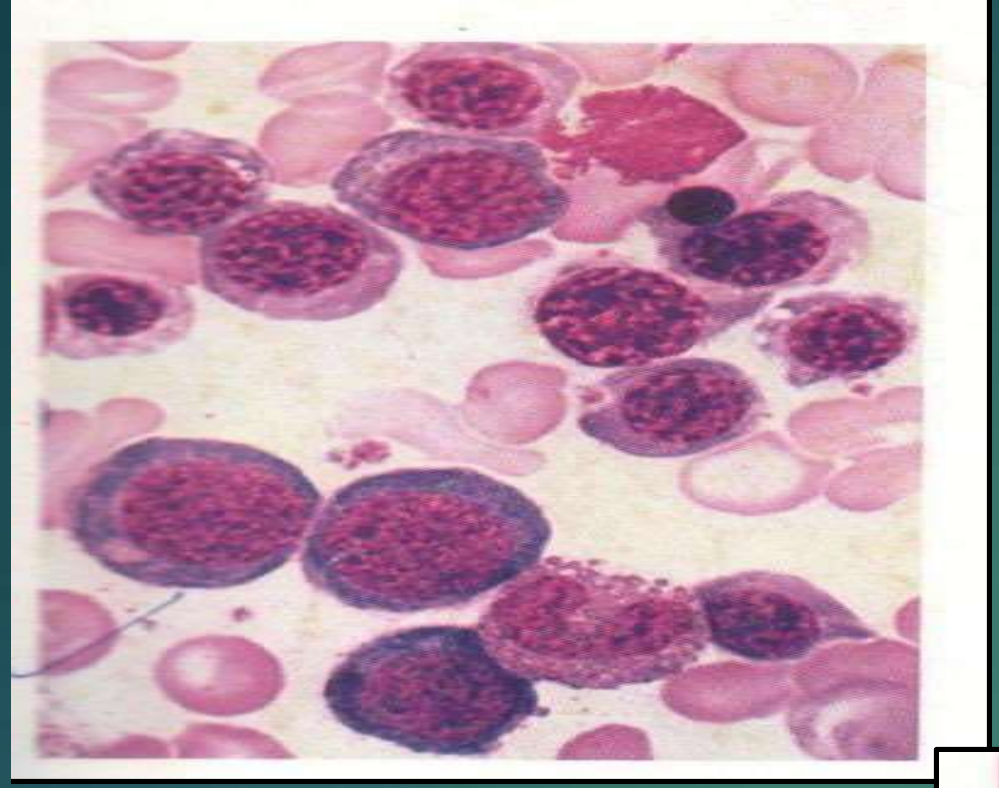
PAS positivity in M6. Not the intense staining of the large abnormal erythroblast.

Erythroid cytochemistry

- Retic stain
- Perl's stain
- Acid-elution test
- Heinz bodis test

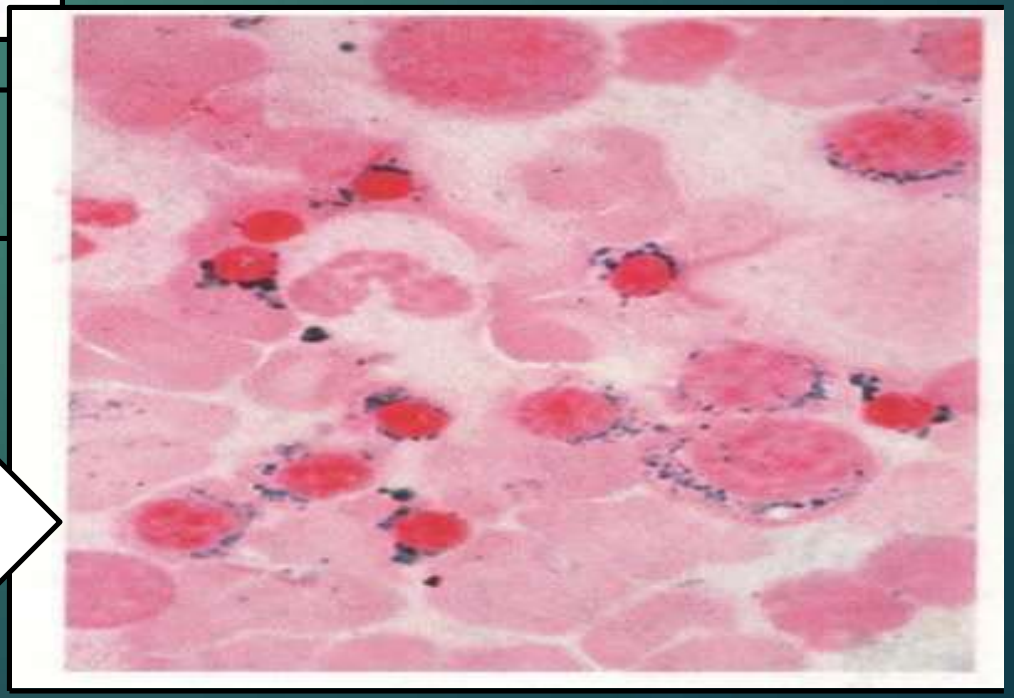


which stain?



Erythroid hyperplasia in bone marrow aspirate smear

Perl's Stain



The presence of **stainable iron** in the bone marrow provides an **indication of iron stores.**

Normally, it occurs as an accumulation of small granules of siderotic material (or **haemosiderin**) lying free and in phagocytes, and some of the normoblasts also contain **1-4 small iron granules ("sideroblasts").**

Iron store **Reduced or absent** in **iron -deficiency anemia**
Increased when there is iron overload**, in infections, dyserythropoietic anaemias, sideroblastic anaemias** and **thalassemia.**

Sideroblastic anaemias	**Perinuclear ring**
Thalassemia	**Large iron granules scattered**
Ring sideroblasts	**Refractory anemia ,MDS**

References :

- Practical Haematology, 12th Edition, Dacie & Lewis
- Haematological Cytochemistry, F.G.J.Hayhoe, Kaplow L S
- ICSHreference method for staining of blood and bone marrow films by azure Band eosin Y(Romanowsky stain). International Committee for Standardization in Haematology. Br JHaematol. 1984 Aug;57(4):707-10

Acknowledgements

Thanks for your attention