Physiology Lab

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The Experiments

- Red Blood Cell (RBC) Count
- White Blood Cell (WBC) Count
- Differential Leukocyte count (DLC)
- Reticulocyte count
- Packed cell volume (PCV)
- Hemoglobin concentration
- Erythrocyte Sedimentation rate (ESR)
- Blood Type
- Bleeding Time
- Clotting Time
- Osmotic Fragility Test

We follow the same procedure, it's the same with some minor differences

Red Blood Cells (RBCs)

which means the center is indented so if we take a cross section of RBC you can see that the central part of it is less thick than the peripheral part

- Normal RBCs are biconcave discs, they have few organelles and no nuclei.
- A major function of RBCs is to transport hemoglobin, which in turn carries oxygen from the lungs to the tissues.
- The average number of RBCs in healthy men is 5,200,000/mm^3 (±300,000) and in healthy women 4,700,000/mm^3 (±300,000)

the number of RBCs is different depending on the age, gender and so on...
so when we take any blood sample, we should give specific information about the person who the blood sample was taken from. is he male or female? how old is he/she? and so on...

- The number of RBCS is regulated within narrow limits, so that oxygen is transported adequately to the tissues and at the same time the cells do not become so numerous that they impede blood flow.

if the number of RBCs falls below normal, this will lead to decrease delivery of oxygen into the tissues, and will lead to a state of hypoxia, on the other hand if the number increases above normal limits, this will lead to increased viscosity of blood, this will impede blood flow within blood vessels, so we should maintain it within normal limits, we have certain mechanisms that help the body maintain it. as we know the life span of RBCs is about 120 days, after that it's removed from the circulation but the BM is always synthesizing new RBCs, so the number of RBCs that enter the circulation equals the the number that's being removed (So the number of RBCs is maintained at a constant normal value)

It's important for us to know the number of RBCs in any blood sample, to define if it's normal or low or high.
The most commonly done test is CBC (it gives us an idea about RBCs (number,characteristics,size,amount of hemoglobin) it also gives us an idea about WBCs (their count, and their types) also about the platelets as well
There are many methods to know the number of RBCs in a blood sample, the method we use in our lab is manual counting, we use a specific type of slide, it's called the meocytometer

- RBC count is typically ordered as a part of complete blood count (CBC) and may be used as a part of health checkup to screen for variety of conditions.

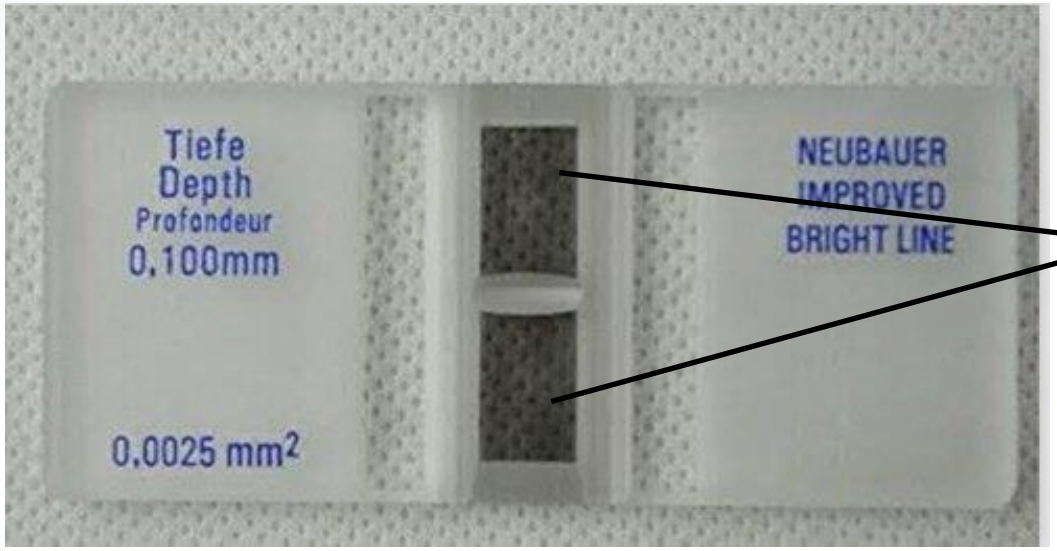
- **Causes of high RBC count (Polycythemia)**

1. Living at high altitudes **Where the Po2 in the atmosphere is low**
2. Cardiac or pulmonary diseases
3. Erythropoietin secreting tumors
4. Smoking.
5. Polycythemia Vera
6. Dehydration

- **Causes of low RBC count (Anemia)**

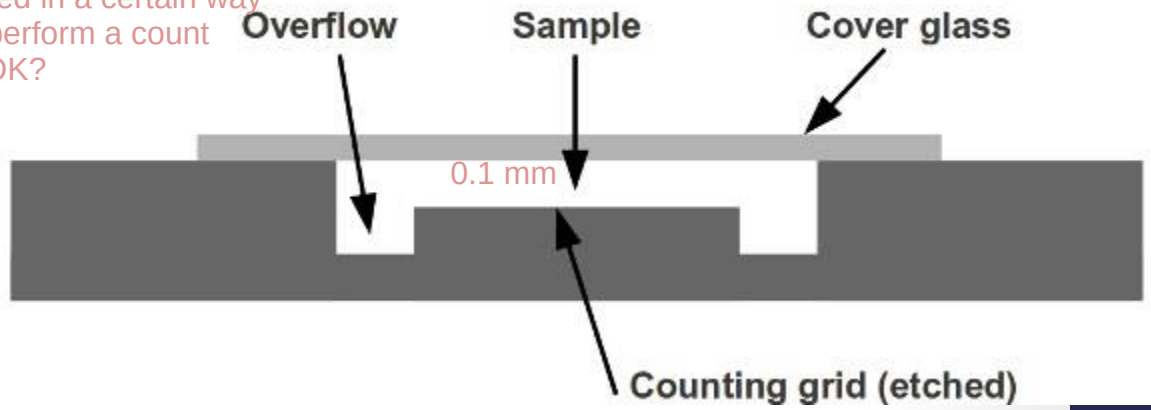
1. Internal or external bleeding
2. Nutritional deficiencies
3. Bone marrow failure **due to chemotherapy, radiotherapy, etc..**
4. Hemolysis of RBCs
5. Chronic renal failure **Synthesis of RBCs in BM depends on erythropoietin which is mainly synthesized in the kidney**

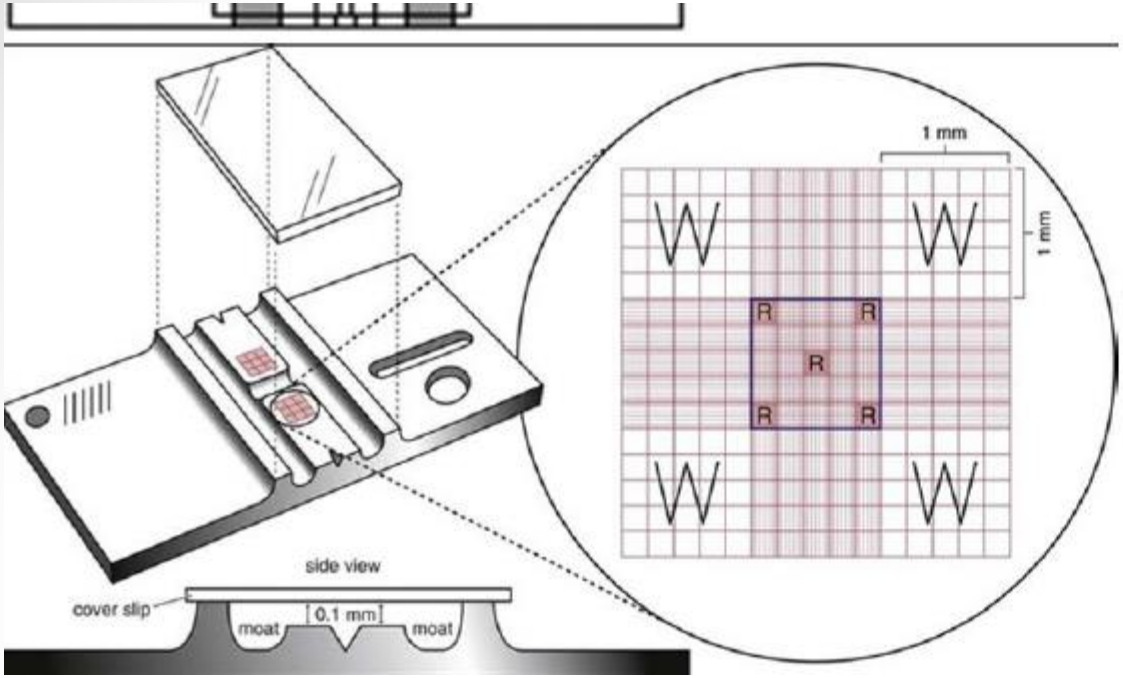
- Hemocytometer is a special microscopic slide that has specific grids engraved on it's counting chamber and is designed to hold a specific volume of fluid.



These two are called counting chambers
We count the RBCs and also WBCs
in one of these chambers.

What's special about this slide is that it's designed in a certain way to hold a specific volume of fluid, so that when perform a count we know the volume of fluid we're counting in. OK?




1 mm
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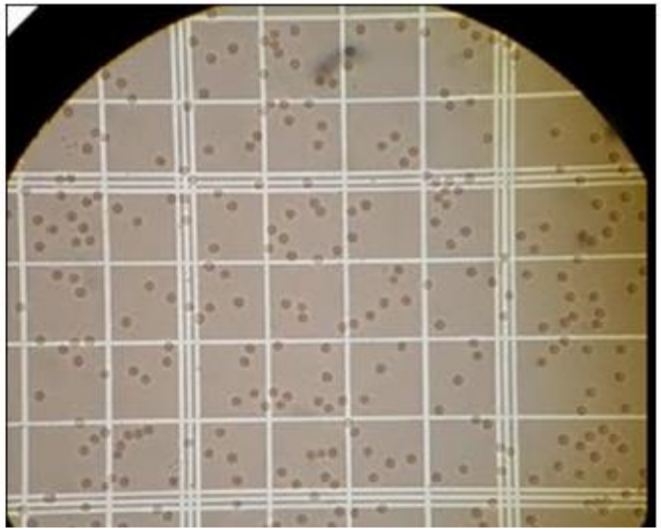
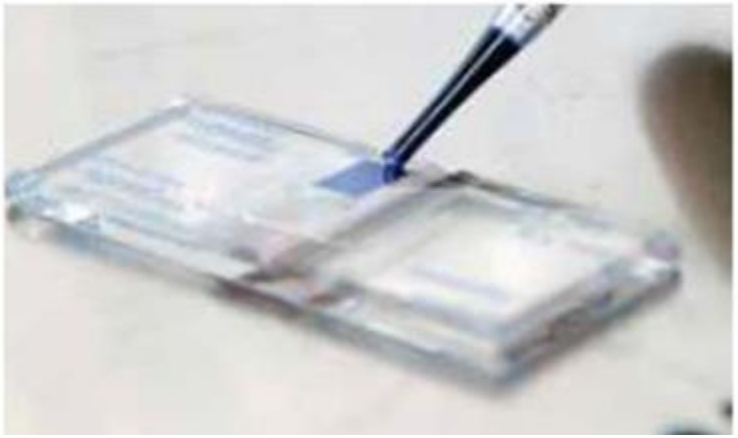
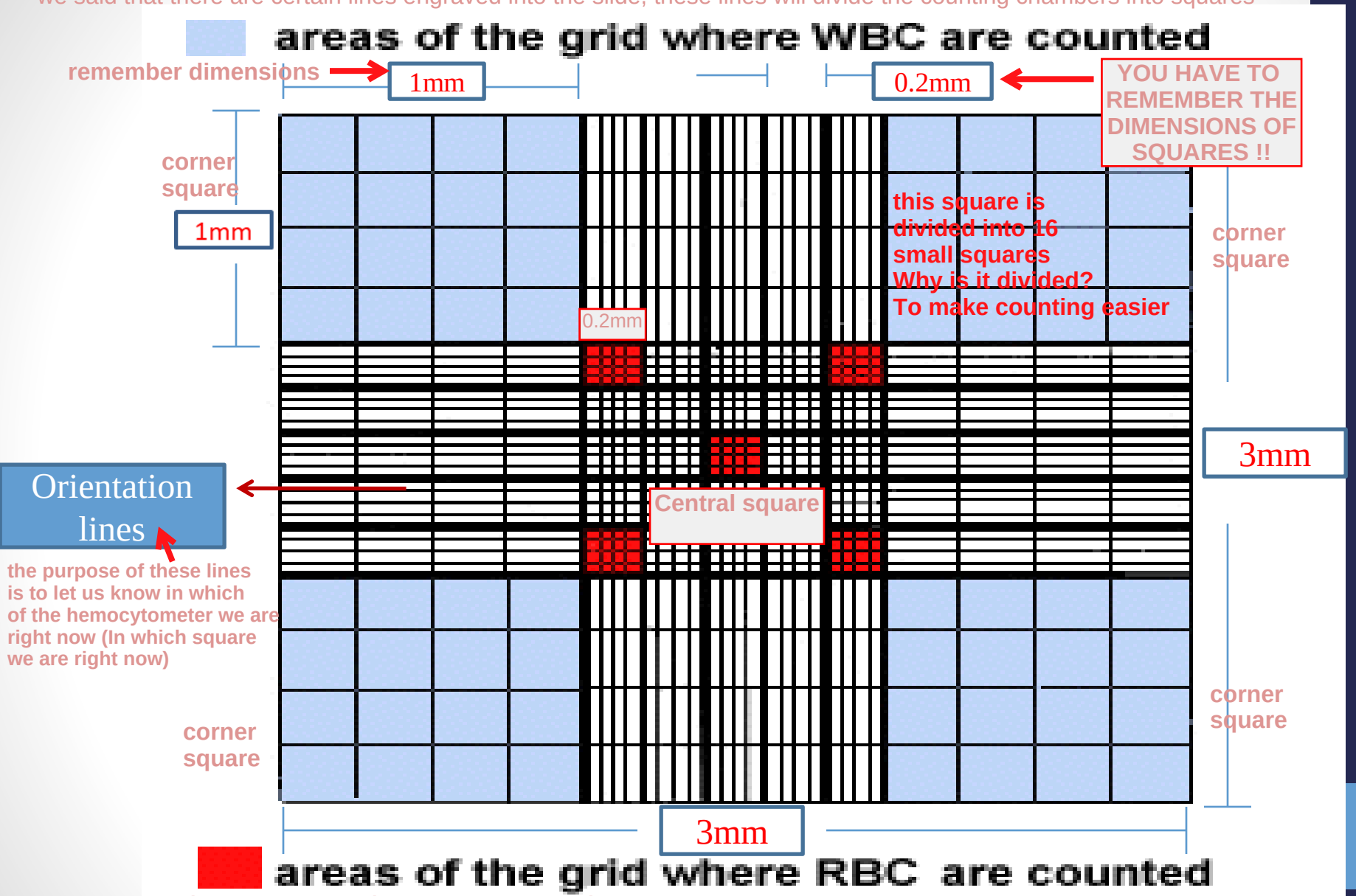




This is how the counting chambers looks under the microscope
we said that there are certain lines engraved into the slide, these lines will divide the counting chambers into squares



*Corner squares: these are squares where WBCs are counted

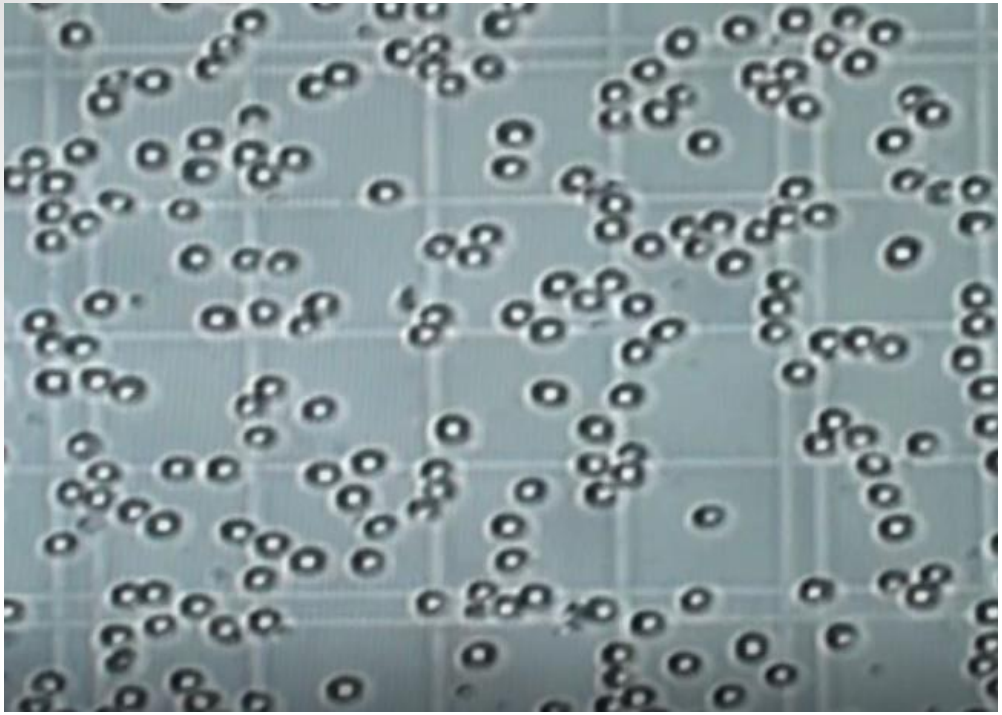
*RBCs are counted in the central square ... the central square is divided into 25 small squares each divided into 16 squares

When we perform RBCs count, we don't count in all 25 squares, because this is gonna take alot of time, we only count in the 4 corner squares and the central one (THE RED ONES)

The procedure

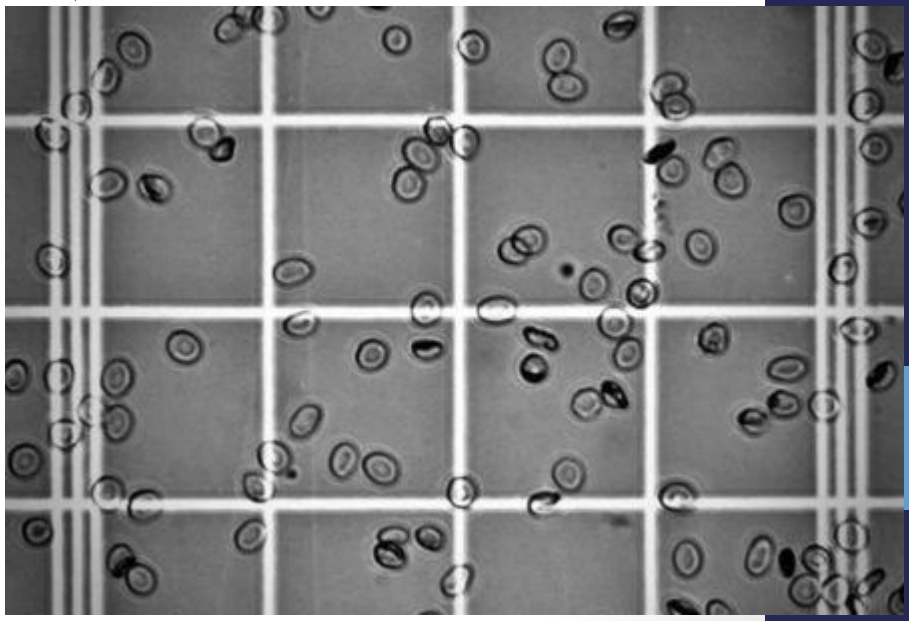
1. Clean the hemocytometer well
   we look at the hemocytometer under the microscope, if it's not clean we might not be able to perform the count properly, so it should be cleaned nicely.
2. Place a coverslip over the counting area. Now the distance between the bottom of the coverslip and the surface of the counting area is **0.1 mm**
   this is the standard, but it might change depending on the circumstances
3. Dilute the blood sample by adding 1 unit of blood to 199 units of an isotonic solvent and thoroughly mix the mixture
   so the cells don't shrink nor swell or rupture
4. Draw a sample using a pipette and gently touch the junction of the coverslip and hemocytometer. The diluted blood will flow by capillary attraction to fill the chamber.
   - ✓ Let it stand for 3 min before you start counting the cells.
     when we first put the sample, the cells will be moving around, then they'll settle
5. Use the 10X lens to identify the center square , then use 40X lens to focus on the smaller squares and count the RBCs (RBCs appear circular in shape with a light center)
   which contains 25 squares
6. Count the number of cells in the five small squares and obtain an average number.

*RBCs under the microscope in the hemocytometer



lines of the squares should be visible

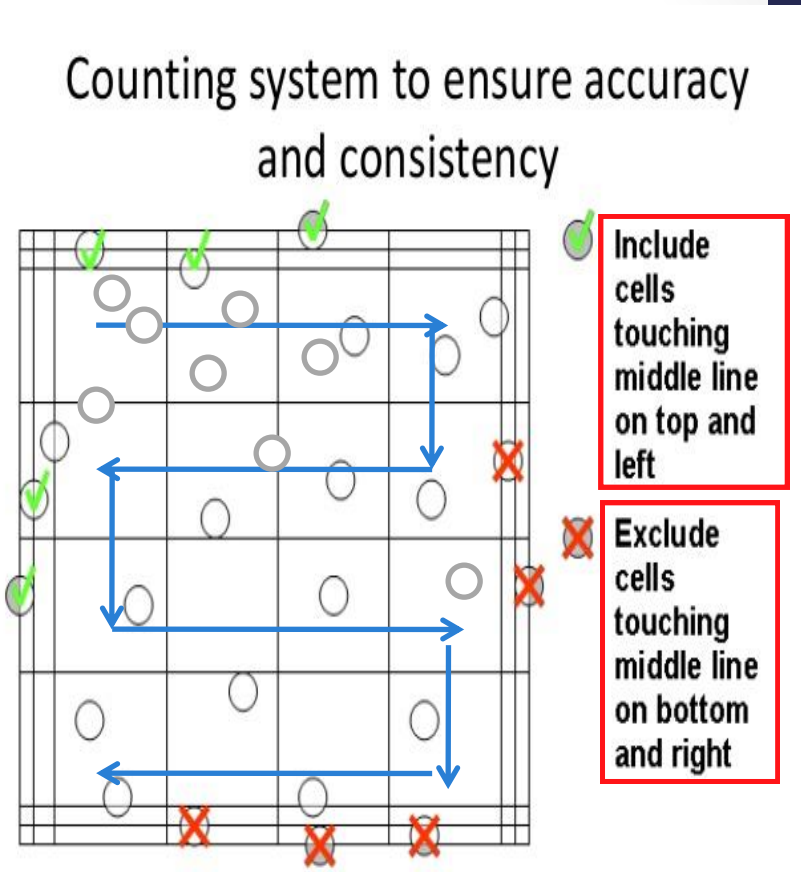
peripheral borders of the square



Count all the cells contained within the square and those touching the upper and right outer lines. The cells that touch the left and bottom outer lines are not to be counted. In each of the four areas, conduct the count in a zig-zag line.

- Start counting from the left to the right and proceed in a zig-zag.

  - To avoid counting the same cells twice, cells that are touching the lines at the tops and left sides of the squares are counted, but cells that are touching the bottoms and right sides of the squares are not counted.



- If the RBC count was (100, 90, 94, 96, 95). What is the RBC count in the blood sample?

1. In this case the average RBC count is 95
2. Dilution factor (DF) = Final volume/ volume of blood
   - Blood is diluted at (1:199) so DF= 200
3. The volume of fluid contained in one small square = (0.2x 0.2 x 0.1)= 0.004 mm^3
   - Volume Correction Factor (VCF)= Desired Volume/ Actual volume
     - Desired volume = 1 mm^3
   - VCF = 1/0.004 = 250

- The number of RBCs in blood sample= The average number of RBCs X DF X VCF = 95 X 200 X 250 = 4,750,000 cells/mm^3

Important Points

- Before you obtain the average number of RBCS make sure the count in the five squares doesn't vary by more than 20 cells.
- If there is a big variation discard the sample from the slide and repeat the experiment.
- DF can change based on the dilution performed during the experiment **Dilution factor is usually 200, unless mentioned otherwise**
- VCF is always the same **= 250**

For example, when you obtain the number of RBCs in 5 squares, you have to look at the highest value you obtained (100, 98,95,90,97,96)
The highest value is 100 and the lowest is 90
it's important that the difference between the highest and the lowest is less than 20 cells otherwise the sample should be discarded

WBC count

- White Blood Cells are part of the immune system
- Move to areas of severe infection or inflammation to provide a rapid and potent defense for the body
- Normal WBC count is 4000 - 11,000 cells/mm^3
- This test is often included in the complete blood count (CBC), it is done to get an impression about the immune system since the Leukocytes (WBC) play an integral role, to get more informative results it is often combined with the differential count

- **Causes of High WBC count (Leukocytosis)**

1. Active inflammation or infection.
2. Certain malignancies
3. Recent vigorous exercise, thermal burn, electric shock, surgery, or trauma.
4. Certain medications e.g. glucocorticoids (neutrophilia)
5. Dehydration.

- **Causes of Low WBC count (Leukopenia)**

1. Bone marrow failure due to aplastic anemia, fibrosis, metastatic cancer, radiotherapy or chemotherapy
2. Autoimmune diseases.
3. Infections like HIV & tuberculosis.

The procedure

1. Clean the hemocytometer well
2. Place a coverslip over the counting area.
3. Dilute the blood sample by adding 1 unit of blood to 19 units of solvent and thoroughly mix the mixture.
   - ✓ The dilution fluid contains an agent (glacial acetic acid) which lyses the red cells, because RBCs count is very high so they might obscure the WBCs so we try to get rid of them.
     It also contains a dye that stains the nuclei of WBCs. This allows a proper count of WBCs.
4. Draw a sample using a pipette and gently touch the junction of the coverslip and hemocytometer . The diluted blood will flow by capillary attraction to fill the chamber.

   ✓ Let it stand for 3 min before you count the cells.

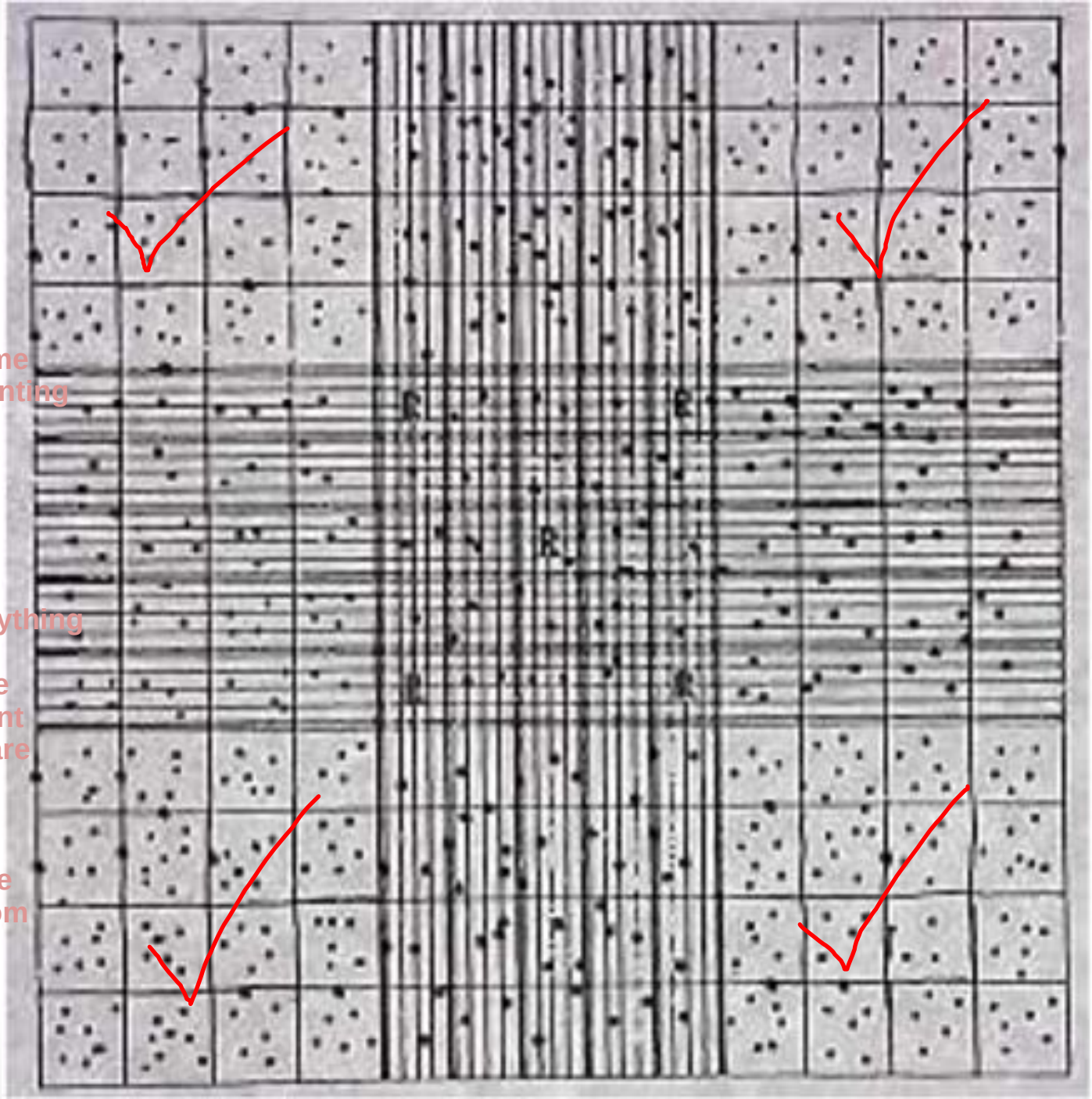
5. Use the 10X lens to count the WBC in the four large corner squares .(WBCs appear as dark dots)

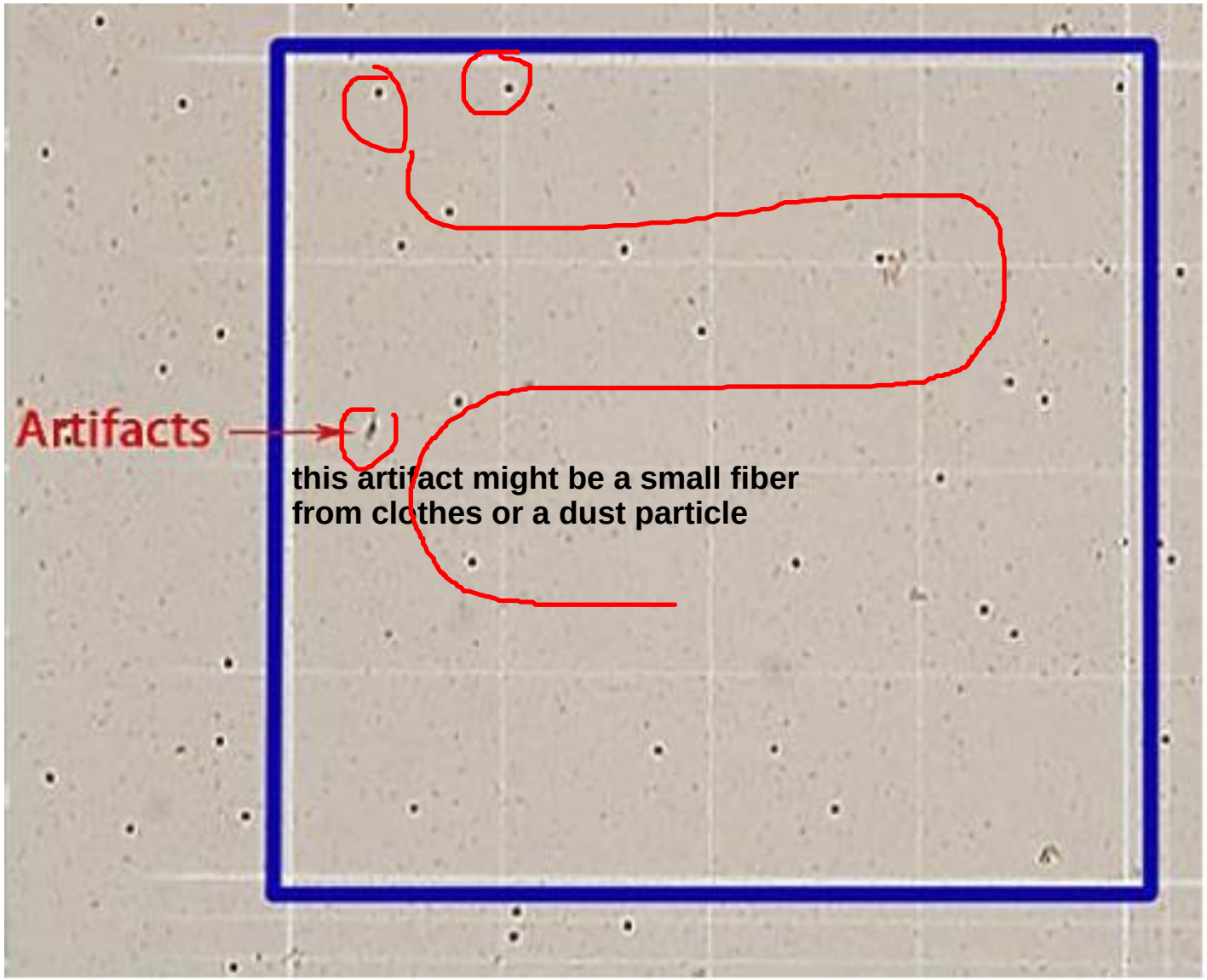
We count the Cells only in the 4 corner squares

we use the same method of counting

from the left to the right in a zig-zag fashion

We count everything inside, the cells on the border we count the ones that are on the left and upper border and we ignore the ones on the right and bottom




Artifacts
this artifact might be a small fiber
from clothes or a dust particle

The calculation

1. Blood is diluted at (1:19) so DF = 20
2. The volume of fluid in the corner square is (1 **X** 1 **X** 0.1= 0.1 mm^3) SO the VCF is 10

✓ If we counted an average of 40 cells in the 4 squares the count of WBCs is….

40 **X** 20 **X** 10 = 8000 cells/mm^3 which is a normal value

•Before you obtain the average number of WBCS make sure the count in the four squares doesn't vary by more than 10 cells

Differential Leukocyte Count (DLC)

- The blood contains 5 different types of white blood cells which are classified into:

1. Granulocytes: have cytoplasmic granules which contain enzymes or chemicals, and have a single multi lobed nucleus (segmented)
2. Agranulocytes: have a single non lobulated nucleus, their cytoplasmic granules are too small to be seen under the light microscope.

It's not enough to know the WBC count only, we have to know what's the number of neutrophils, eosinophils, basophils and so on.

1. Neutrophils: 40-80 %
2. Eosinophils: 1-4 %
3. Basophils: < 1%

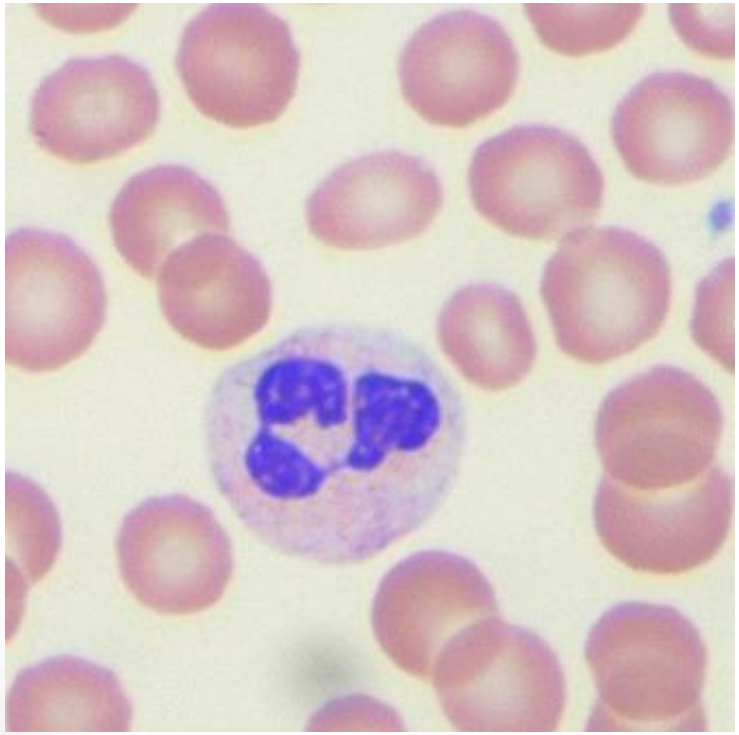
Granulocytes

4. Lymphocytes: 20-40%
5. Monocytes: 2-8%

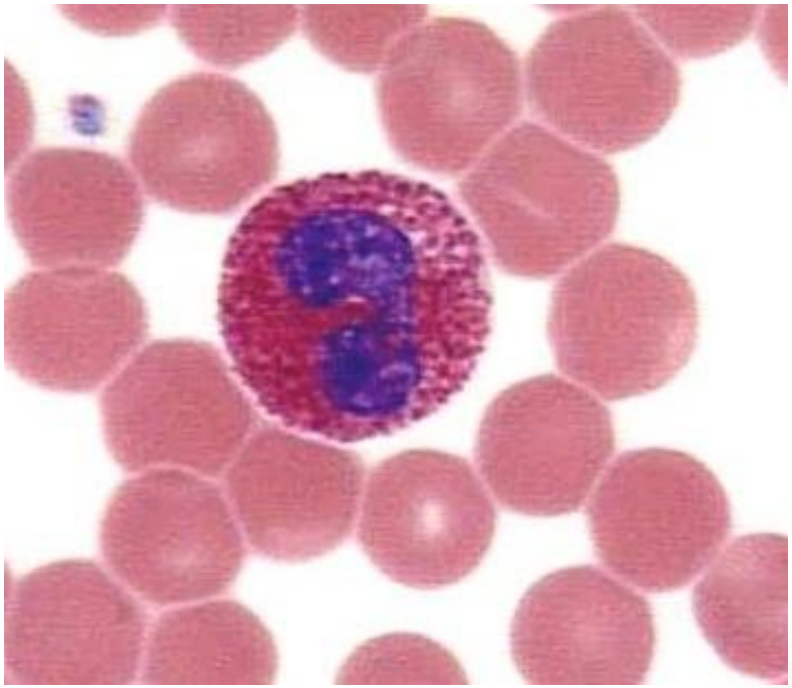
Agranulocytes

Neutrophils have nuclei with several lobes and fine pink granules in their cytoplasm.

They are called neutrophils, because their granules are not very amenable to staining with either acidic or basic dyes

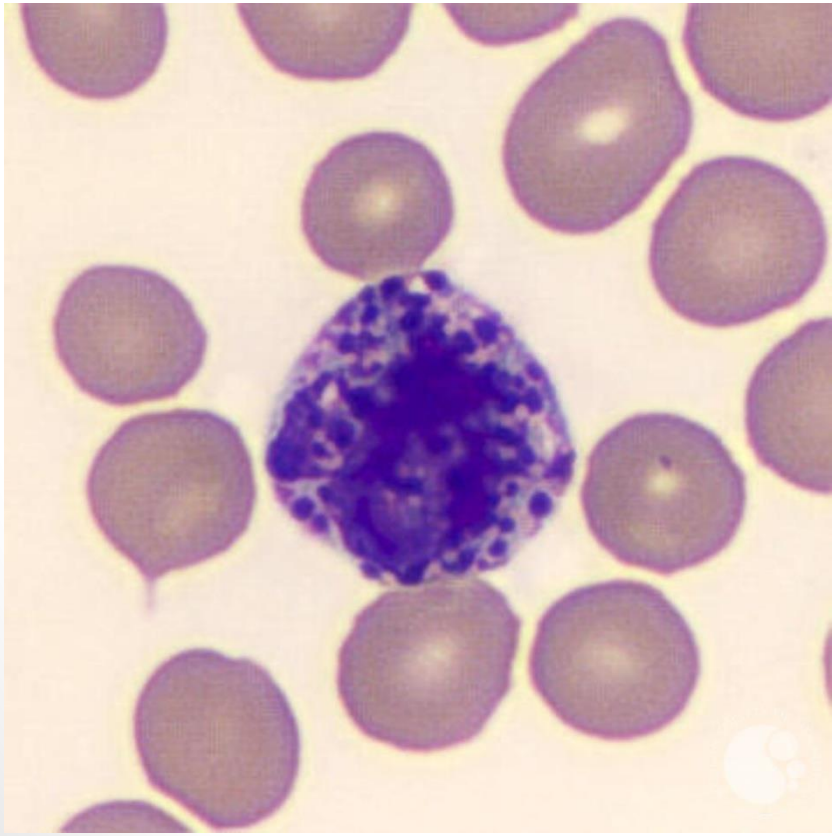


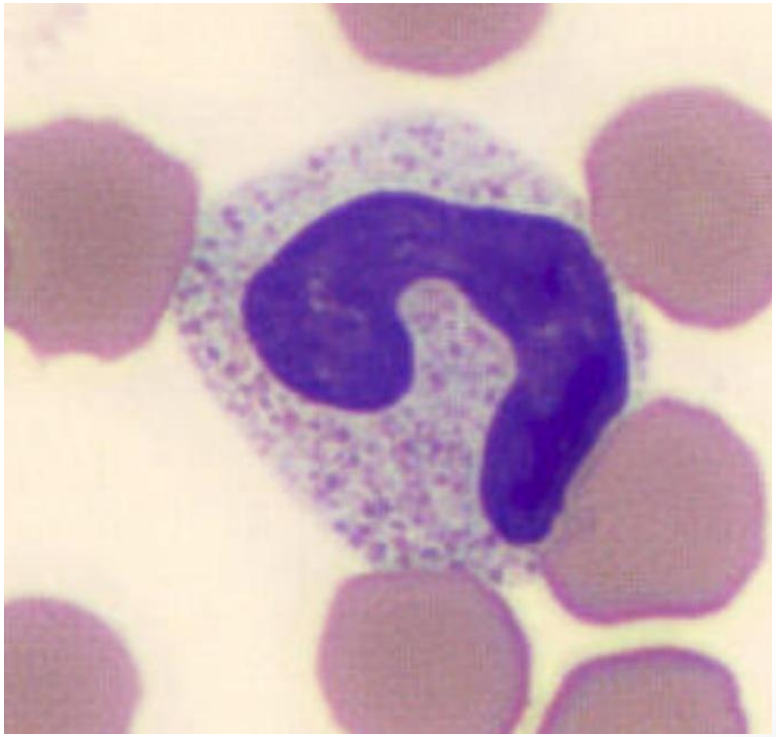
Eosinophils have bi-lobed nuclei and medium-sized granules that can be stained bright red with an acidic dye.



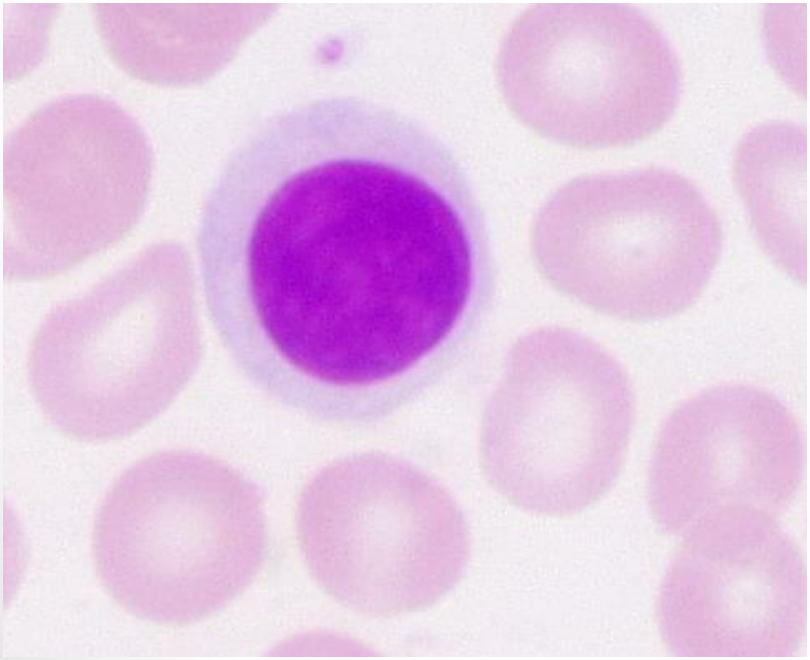
Basophils have bi-lobed or S shaped nuclei and large granules which stain dark blue with basic dyes and completely obscure the nucleus

Neutrophilic Band cells are immature neutrophils, usually make less than 5% of the total WBC count, their nucleus isn't segmented

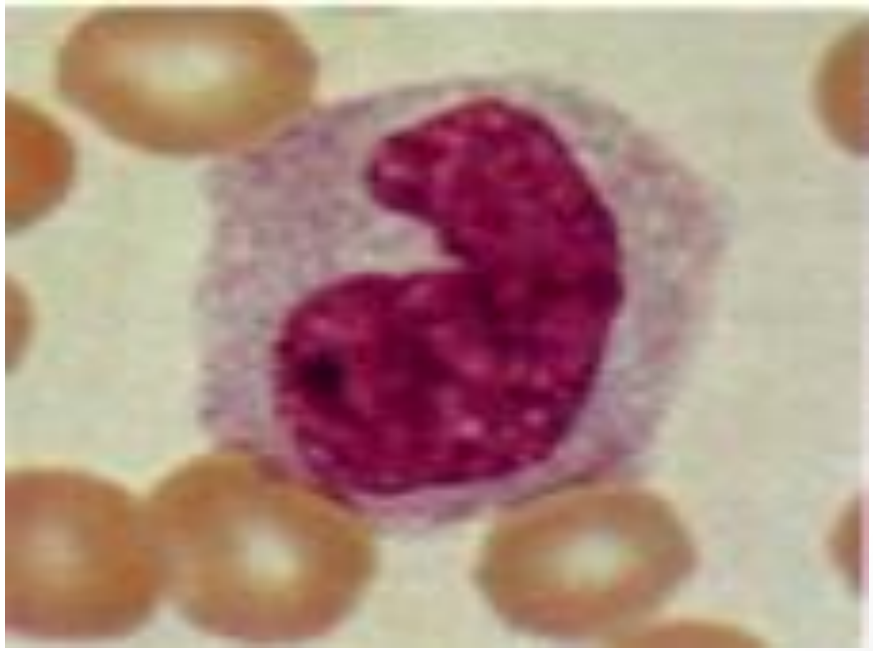


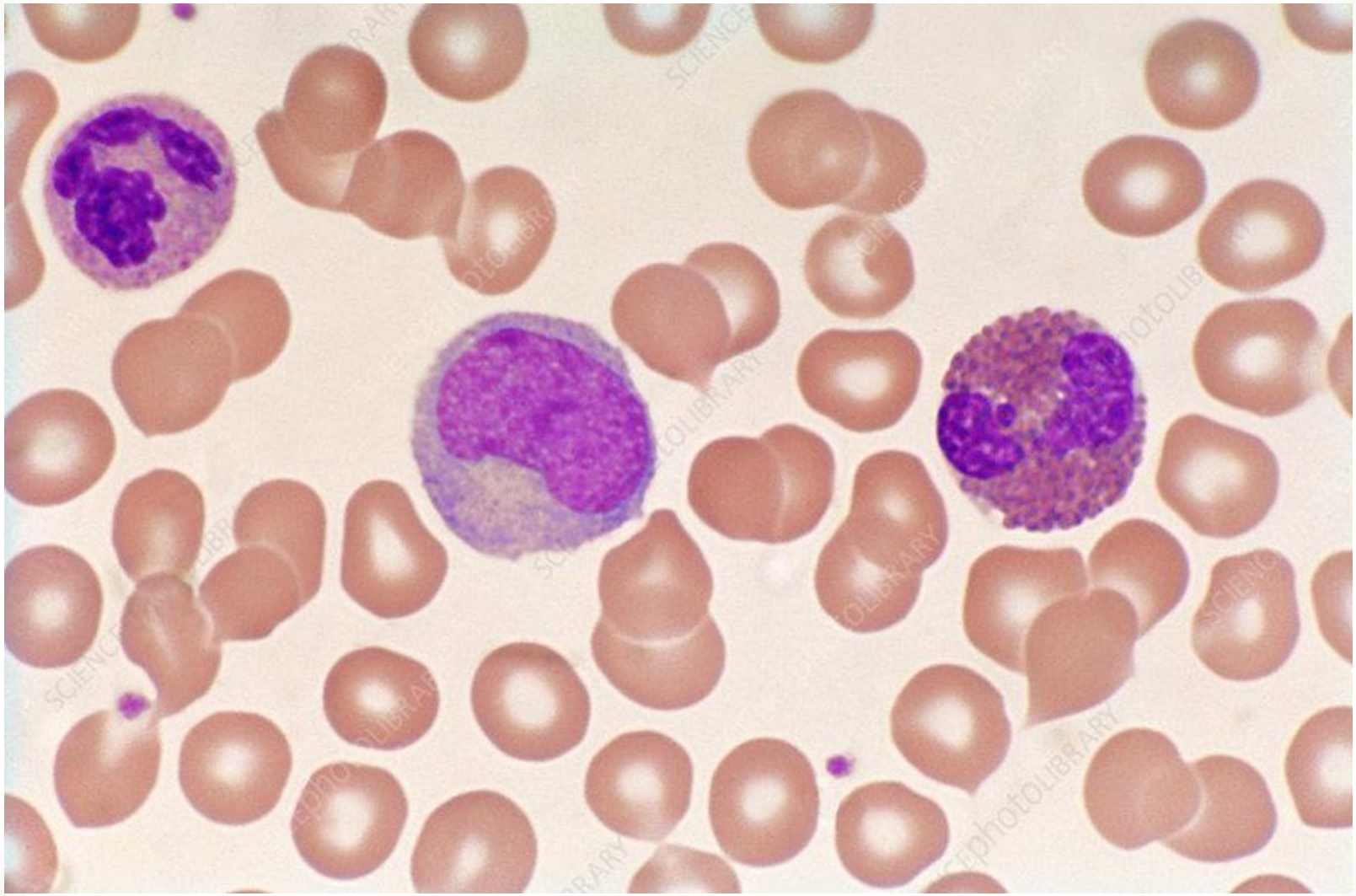


- **Lymphocytes** have a very large nucleus taking up most of the cytoplasm. The cytoplasm has no granules. Most cells are small in size.
- We can’t differentiate between the B and T lymphocytes under the light microscope.



- **Monocytes** are large cells. They have large indented nuclei, often kidney-shaped. Their cytoplasm has fine purple granules which give it a "ground glass" appearance.

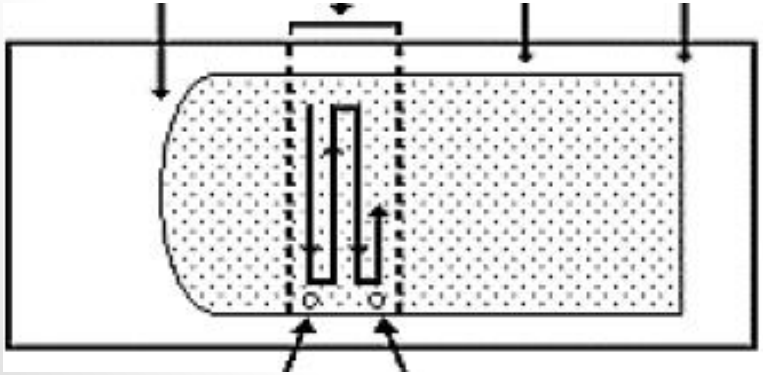




The procedure

1. A drop of blood is thinly spread over a glass slide, air dried, and stained with an acidic dye (red) and a basic dye (blue-purple).
2. The slide is examined under a microscope using an oil immersion lens.
3. Two hundred white cells are then counted and classified.
4. The number of each type of cells is expressed as a percentage.

- To do this one must be able to distinguish between the 5 types of WBCs



N	60%	60
L	11	30
M	1	5
E	1	3
B		2

Importance of DLC

- Gives relative percentage of each type of WBC
- Helps reveal the presence of abnormal WBCs like blasts or lymphoma cells.
- Used along with WBC count to generate an **absolute value** for each type of WBCs. absolute values are always better especially for monitoring some malignancies or following up chemo patient
  - Relative percentages can be misleading
  - Absolute values are also useful for monitoring certain conditions.
  - Absolute count =WBC (cells/ μL) x percent of the specific WBC type ÷100

Absolute count calculation

- If the WBC count is 6000 cells/mm^3 and the lymphocytes make 30% of the DLC, the Absolute lymphocyte count (ALC) will be:

WBC count **x** (Lymphocyte%)/100 =

(6000 X 30)/100 = 1800 cells/mm^3 **This is the lymphocyte**

The same thing applies for eosinophil,basophils and monocytes except for neutrophils (We have to include neutrophilic band cells)

• Absolute neutrophil count (ANC)=WBC (cells/ μL) x percent (neutrophils + neutrophilic band cells) ÷100

1. Neutrophilic leukocytosis: is defined as a total WBC above 11,000/μL along with an absolute neutrophil count (ANC) greater than 7700/μL
   - Bacterial infections, inflammatory conditions, stress.

2. Lymphocytic leukocytosis : is defined as a total WBC above 11,000/μL along with an absolute lymphocyte count greater than 4500/μL
   - Viral infections as infectious mononucleosis, mumps, rubella and pertussis or in acute and chronic lymphocytic leukemias.

3. Monocytic leukocytosis:
   - Acute or chronic bacterial infection and chronic inflammation

4. Eosinophilic leukocytosis :
   - Parasitic infections & allergic conditions

6. Basophilic leukocytosis:
   - Allergic conditions

7. Neutropenia : absolute neutrophil count is less than 1,500 cells/ mm^3
   - Certain infections like typhoid fever, HIV & CMV, chemotherapy, radiotherapy, and autoimmune diseases.

8. Lymphocytopenia:
   - May occur in the normal elderly or be associated with chronic infection or malignancy.

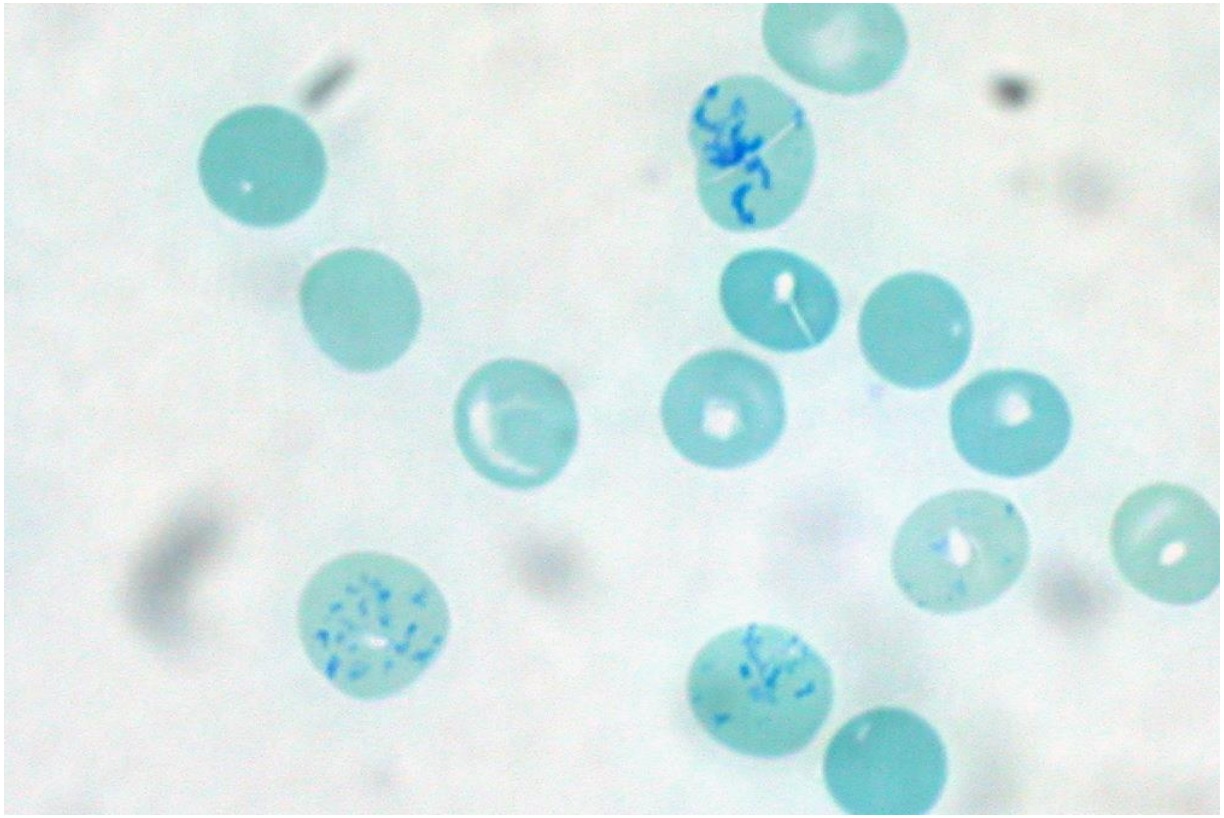
Reticulocyte Count

- Reticulocytes are the immediate precursor of RBCs, following their release to the blood stream they mature within 1-2 days into RBCs.
- Contain a small amount of basophilic material, mainly remnants of the Golgi apparatus & mitochondria

They're slightly larger in size

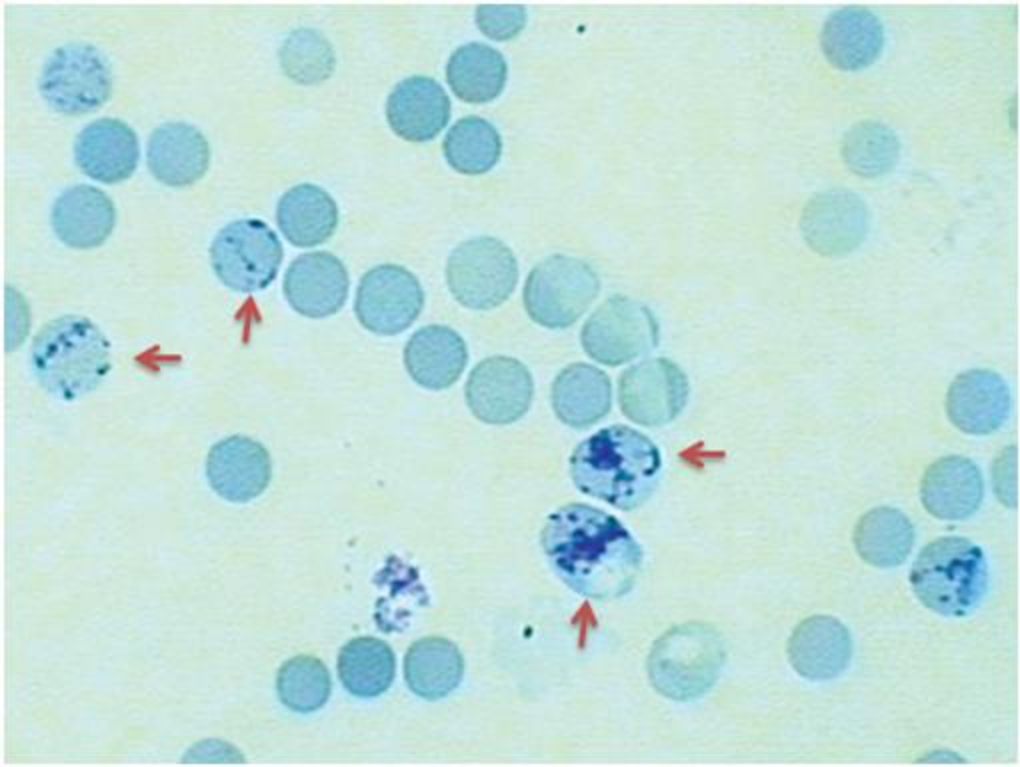
- They normally make less than1-2% of all RBCs
- Used to estimate the degree of effective erythropoiesis
- Their number increases in cases of bleeding and RBC hemolysis and decreases in cases of bone marrow failure

If supravital staining (new methelene blue) is performed on a blood smear, the reticulocytes appear larger than RBCs and contain dark blue dots and curved linear structures in their cytoplasm (remnants of ribosomes).



The procedure

- 500-1000 RBCs should be counted and the number of reticulocytes noted. The count is expressed as a percentage which can be used to calculate the absolute reticulocyte count (ARC) .



Absolute reticulocyte count

- ARC accurately reflects the degree of reticulocytosis regardless of the degree of anemia. The normal absolute reticulocyte count is between 25,000 to 75,000/mm^3

- ARC = (RBC count X reticulocyte%)/100

Reticulocytosis and Reticulocytopenia

- Condition associated with an increase in reticulocytes:

Because when we have hemolysis of RBCs, the BM is trying to make more RBCs and the number of Reticulocytes will be higher than normal

  - Hemolytic anemias: Immune hemolytic anemia, RBC membrane defects, Sickle cell diseases,
  - Following hemorrhage
  - Following treatment of anemias

- Condition associated with a decrease in reticulocytes:
  - Iron deficiency anemia
  - Aplastic anemia
  - Radiation therapy
  - Tumor in bone marrow

That's why it's important to know the reticulocyte count, we can express it as a percentage, but it's better to have an absolute value which is between 25,000 and 75,0000/mm^3