Lab: Blood Smear and RBC Count

Aim: Learn to count cells, observe and identify different blood cells in a smear, quantify their proportions and count RBCs per µl (mm^3) using a haemocytometer.

Introduction:

The technique of making a peripheral blood wedge slide (or push slide) was developed by Maxwell Wintrobe[1]. Hematology is the study of blood and the blood smear one of the most basic and yet most reliable ways of evaluating blood for multiple conditions of disease.

We will use a droplet of blood to make a **thin smear**, dry it, fix and stain it and observe under a microscope. Cell fixation is done by placing the slide in methanol (CH3OH). It works by precipitating proteins and carbohydrates. Additionally it also dehydrates the sample. The action on lipids is thought to involve dissolution. It is expected by dehydration the cells undergo some amount of shrinkage. Staining by a combination of acidic (cytoplasm label) and basic (DNA label) dyes leads to very good contrast images of not just red blood cells (RBCs) but also white blood cells (WBCs) of various types.

The **Hemocytometer** is a classic device used to measure cell numbers, particularly in blood samples. Counting is performed by introducing citrated (4% w/v sodium citrate (dihydrate), pH adjusted with citric acid, USP) blood into the counting region of the chamber (Figure 1). The height of the chamber is 0.1 mm. Using this we can estimate the volume occupied in the boxes marked R (for RBC). The total length of one side of 5 R-boxes is 1 mm. Using this measure and a mean count of cells in each R-box, we can estimate the number of RBC's in a unit of blood as follows:

$$C_{RBC} = \frac{\langle N_{RBC}^{R-box} \rangle \cdot 25}{V_{R25}} \cdot d_f$$

(Eq. 1),

C_{RBC}= RBC count (cells/µl)

$\langle N_{RBC}^{R-box} \rangle$ = Mean RBC-count from five R-boxes (usually the 4-corners and central)

V_{R25} = Vol. of the 5x5 RBC region in µl

d_f =Dilution factor

This count has been shown to vary between men and women. We will take one sample each to test this. Counting is done by eye.

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[1] Wintrobe MM. (1932) The size and hemoglobin content of the erythrocyte. Methods of determination and clinical application. J Lab Clin Med. 115(3):374-87. (Reprinted 1990)

When counting certain conventions need to be used. **Cells at edges of a line** are counted only in the L-shape, i.e. lower line and left-lines. This reduces over-counting artefacts. Averaging over 4-5 R-boxes ensures inhomogeneities in
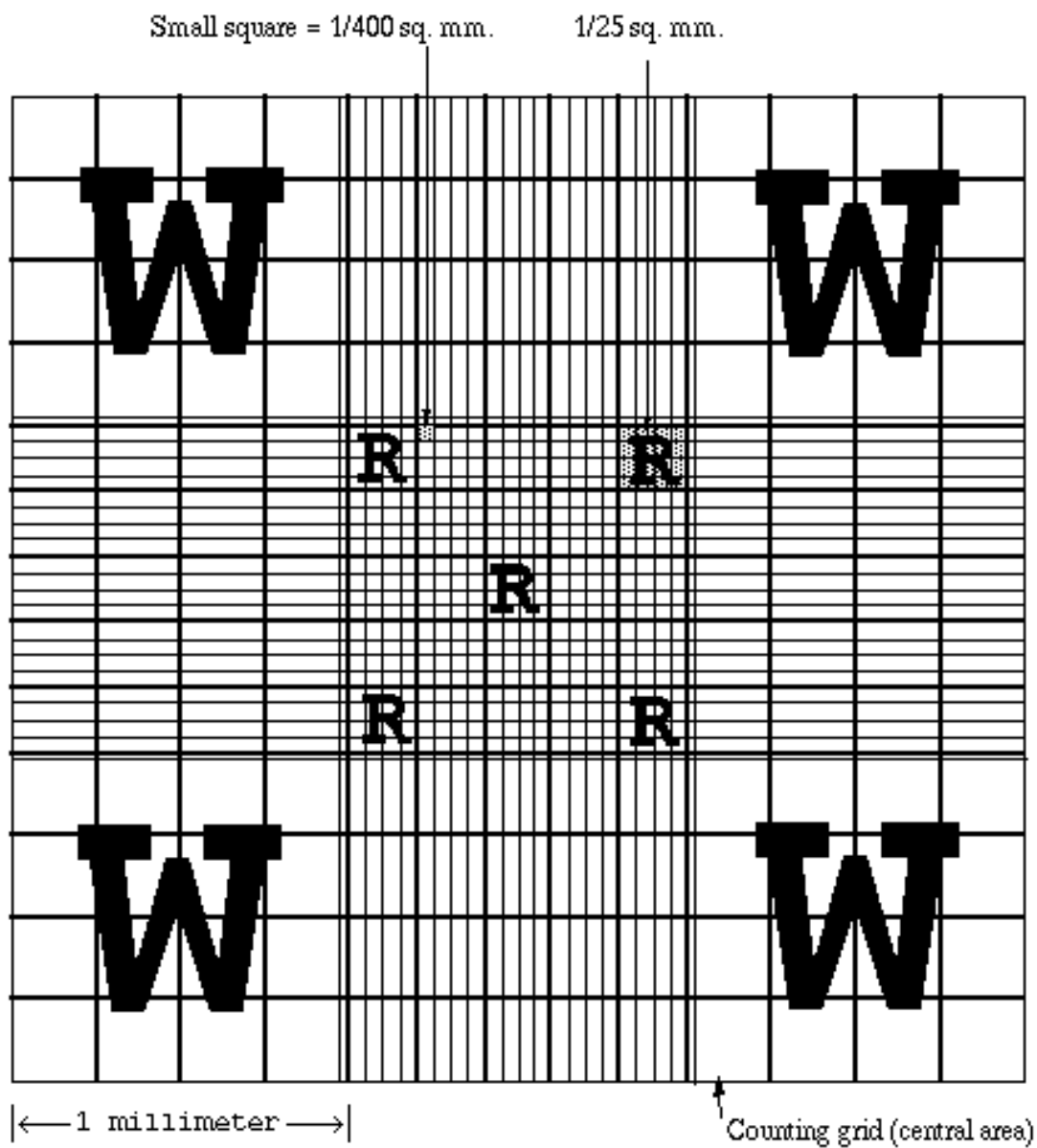
spreading or clumping of cells don't affect the final result.

Figure 1: Top view of the chambers of a Neubauer's Hemocytometer for RBC (R) and WBC (W) counting with scales indicating sizes of each region. The height in the z-direction of the entire chamber is uniformly 0.1 mm.

Materials:

Biologicals:
1. Droplet of blood from capillary bleed

Glass/plastic ware:
1. RBC diluting pipette with hose and bulb
2. 50 ml beaker for waste material

3. Trash bin for lancets
4. Plastic droppers

Chemicals
1. RBC diluent 3.2 or 4% w/v Sodium Citrate ($Na_3C_6H_5O_7$)
2. A 2.5% bleach mixture for cleaning
3. 95% Alcohol for rinsing
4. 99% Methanol for fixation
6. Giemsa stain (1:20, vol/vol from stock)

Intruments
1. Sterile lancet
2. Hemocytometer for RBC and WBC counting
3. Microscope

Others
1. Tissue paper
2. Gloves for use while staining

METHOD

A. Making blood smear
1. Place a clean slide on a piece of tissue paper and **write your unique initials** in one corner, using the glass-marker pen.
2. Using a cotton swab dipped in 75% ethanol, clean the middle- or ring-finger with it. This both disinfects the surface as well as causes a slight increase in blood flow due to the evaporation and resultant compensatory blood-rush.
3. Carefully open the protective covering of a sterile Stab gently (vertical w.r.t. the length of the finger) the finger-tip and wait for a drop of blood to come up.
4. Make this drop fall ~1 cm from one of the short-edges of a clean slide.
5. Using the other slide, make contact at 30-40 degrees with the slide on which the blood droplet is. Drag the droplet towards the other end. As a result you should have a comet-like appearance of the blood smear.
6. The smear should have a 'comet' like appearance- thick initially and becoming very thin at the end. The comet tail area is the one we will observe under the microscope.

B. Staining the smear
1. Fix the smear in ~99% **methanol 3-5 minutes** by dipping in Coplin Jars.
2. Stain in **Giemsa** (methylene blue and eosin mixture) by dipping in Coplin Jar containing stain for a total time **~15 minutes.**
3. **Rinse** the slide with **tap water** at room temperature (ensure rinsing doesn't wash away your sample).
4. Drain off the water by leaning it at ~45 degrees and leave it to air-dry.

C. Microscopy
Observe under 10x magnification to see that the cells are stained. Move to the 40x lens to see further details (be careful to prevent lens and slide collisions). Erythrocytes are the most numerous cells with a diameter of ~6-7 micrometers. Some larger macrocytes (d>9 um) and smaller microcytes (d<6 um) have been seen. Purple diskettes around 3 um diameter are platelets. Leukocytes show nuclear stains purple in colour. The different granule patterns and nuclear morphologies allow a classification.

D. Counting RBCs (using blood donated to you)
1. As before make a pin-prick using a fresh unused lancet on the index- or ring-finger. If you have been pricked before on that finger, use another finger.
2. Bring a cleaned RBC dilution pipette tip close to the droplet of oozing blood. Using the bulb allow (by capillarity and pressure) blood to enter up to the 0.5 mark.
3. Using a small tube of Sodium-Citrate solution, additionally aspirate this solution to reach the mark 101 (1:200 dilution).
4. Gently turn the dilution tube in your hand.
5. Introduce the diluted blood in the Hemocytometer.
6. Count as per the instructions in the finer grid.

OBSERVATIONS
Answer these questions in your experimental write-up in the **lab note-book**:

How many morphologically different cell types do you observe?
What is the ratio of number of RBCs compared to WBCs you observed on your slide?
Does this ratio match with what people expect from more careful measurements? If not, what could be the potential reasons for the deviations?
What mean count do you get for RBCs/µl (µl=microlitre)? Is it different for the sample from a man compared to a woman?
What are the likely sources of error in counting?

Please paste **this** hard copy of the protocol into the lab notebook.

References:
1. Riley, Watson, Sommer, Martin. How to Prepare & Interpret Peripheral Blood Smears
http://www.pathology.vcu.edu/education/PathLab/pages/hematopath/pbs.html#Anchor-The-49575
2. Complete blood count, MedLine Plus, NIH, USA.
Link: http://www.nlm.nih.gov/medlineplus/ency/article/003642.htm
3. Gretchen L. Humason, W.H. Freeman. Animal Tissue Techniques *(fixation and staining protocols)*
4. Johnson, Johnson, Timmons and Hall (2002) Essential Laboratory Mathematics: Concepts and Applications for the Chemical and Clinical Technician. Cengage Learning.
5. Ichihashi et al. (1996) The Haematology Atlas, Nagoya University School of Medicine, Japan. http://pathy.med.nagoya-u.ac.jp/atlas/doc/index.html

RBC count in the 16 boxes

Name of donor (whose blood was counted):
Sum count in 16 boxes:
Mean count for R-boxes (5):
Standard deviation:

RBC Count: