Name: **Roll No.:**

Lab: Blood Smear and RBC Count

Aim: (a) Identify blood cell types in a smear and quantify their proportions.
(b) Quantify RBC concentration in blood by counting in a haemocytometer and estimate errors in estimation.

NOTE: **Please paste/staple this protocol into the lab notebook.**

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. Cells are fixated by immersion in methanol (CH_3OH), which precipitates proteins and carbohydrates. Additionally it also dehydrates the sample. The action on lipids is thought to involve dissolution. Due to dehydration, the cells are expected to shrink to a small extent. 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:

$$C_{RBC} = \frac{\left\langle N_{RBC}^{R-box} \right\rangle \cdot 25}{V_{R25}} \cdot d_f \qquad \text{(Eq. 1),}$$

C_{RBC}= RBC count (cells/μl)
$\left\langle N_{RBC}^{R-box} \right\rangle$= 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.

[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 inhomogeneity in cell spreading or clumping doesn't affect the final result.

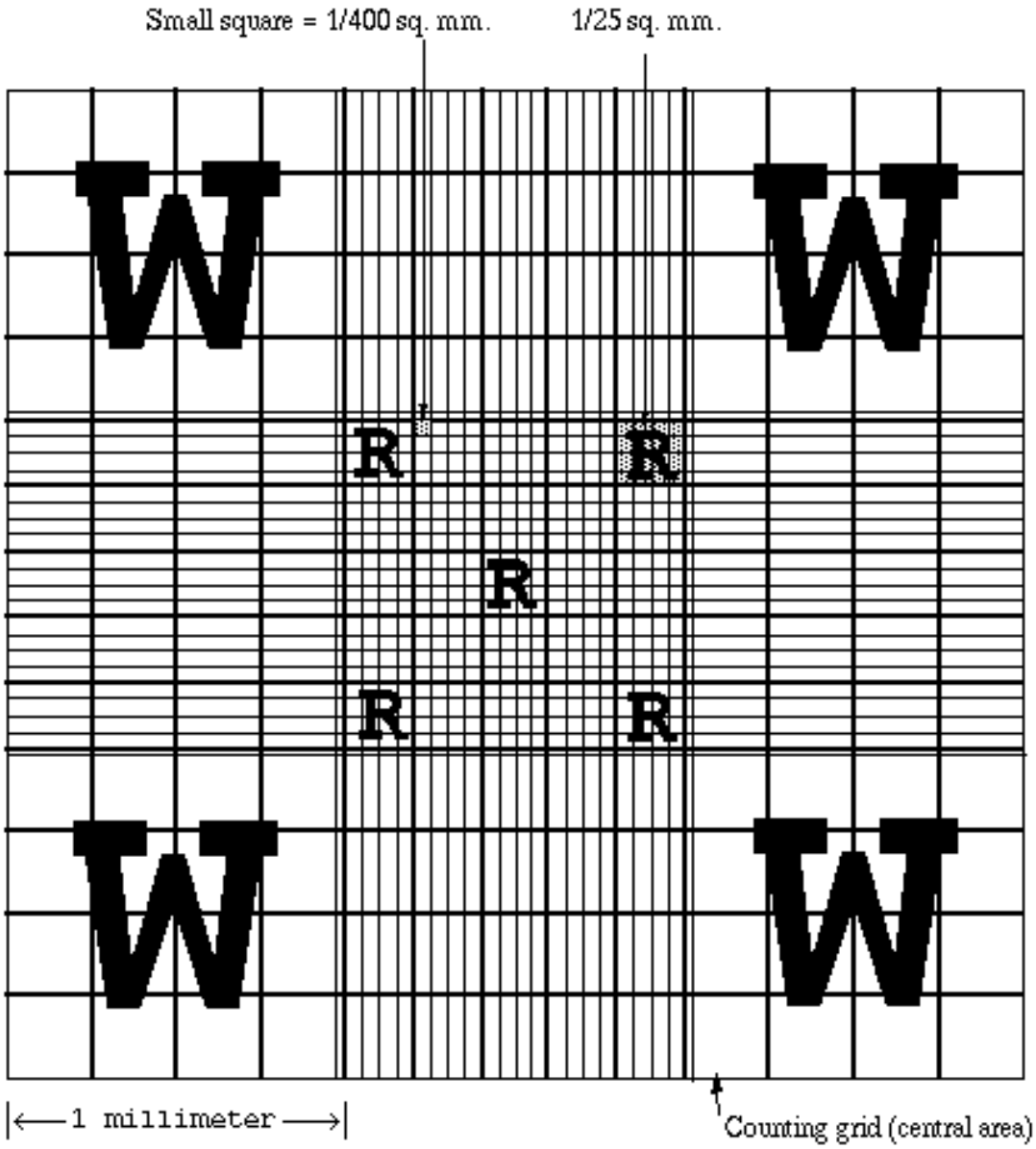

Figure 1: Top view of the chambers of a Neubauer's Hemocytometer for RBC (R) and WBC (W) counting with scales indicating sizes of each region. The height in the z-direction of the entire chamber is uniformly 0.1 mm.

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)
7. Bleach to decontaminate all material coming in touch with blood

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 a glass-marking 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 **~5 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)

The blood-sample preparation will be demonstrated, and your task is to note observations. Using a pin-prick with a fresh unused lancet on the ring-finger (use an alternate finger if already pricked). 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. Using ~0.5 ml of Sodium-Citrate solution, aspirate to reach the mark 101 (1:200 dilution). Mix by gently turning the dilution tube by hand and introduce diluted blood in the Hemocytometer.

1. Count cells in the finest grid. Each of you must have a readong in a 16x16 grid.
2. Use the average of three readings to calculate RBC count per ml.

OBSERVATIONS

Note in your lab note-book (and for assessment):

1. Name and quantify the number and types of WBCs observed in the Thin Smear.
2. Estimate the ratio of RBCs:WBCs in your smear from your WBC and RBC counts.
3. Does this ratio match with expected values? If not, discuss the reasons for the deviation?
4. What mean count do you get for RBCs/µl (µl=microlitre)? What is the standard deviation (s.d.)?
5. What are the expected mean RBC counts from men and women. Does the difference still hold despite counting error (e.g.: s.d.)?

Further reading: The source of errors and their statistical meaning from sampling theory were discussed in papers by Student (6) and Fisher (7).

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
6. Student. (1907) On the error of counting with a haemacytometer. *Biometrika* 5: 351-360. http://www.jstor.org/stable/pdfplus/2331633.pdf
7. Fisher RA. (1939) Student. *Annals of Eugenics* 9: 1–9.

RBC count in the 16 boxes

Name of donor (whose blood was counted):
Sum count in 16 boxes:
Mean count from n=5 RBC containing boxes (use data recorded by others alongwith your own):
Standard deviation:

RBC Count: