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Ghai's Textbook of

PRACTICAL PHYSIOLOGY



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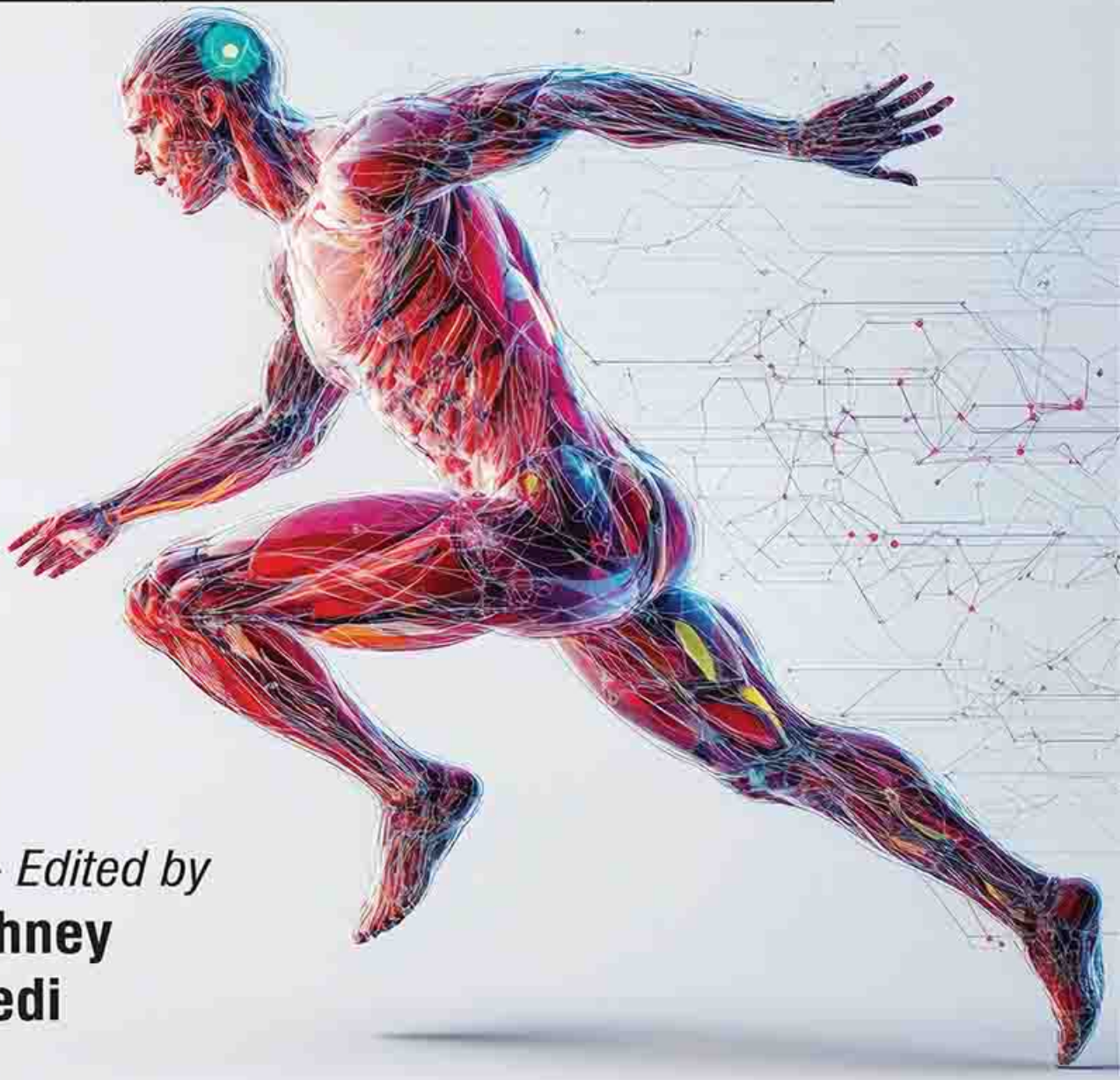
11th Edition



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SECTION 1

Hematology

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1.1: THE COMPOUND MICROSCOPE

STUDENT OBJECTIVES

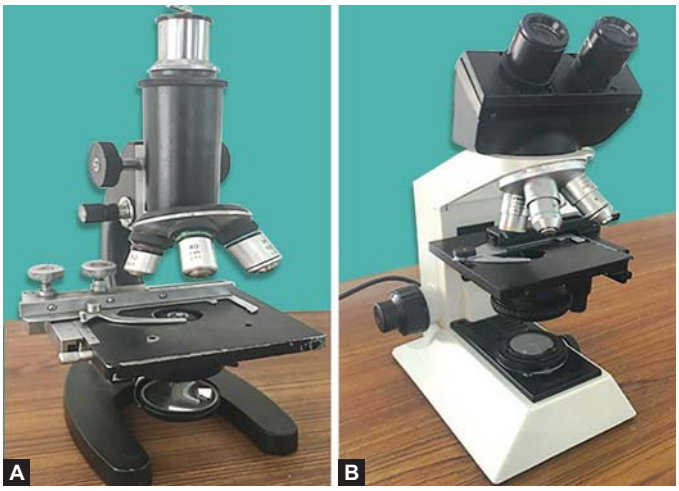
After completing this experiment, the student should be able to:

- Name the different parts of the microscope and explain the functions of each.
- Explain the physical basis of microscopy and define the terms: magnification, resolution, and numerical aperture.
- Describe the mechanism of image formation and the type of image seen.
- Explain how to use low power (LP), high power (HP), oil immersion (OI) objective to obtain different magnifications.
- Describe the procedure (protocol) that must be followed every time while using a microscope.
- Name the precautions that must be observed during and after using the microscope.
- Explain the basic working of other types of microscopes.

INTRODUCTION

Compound microscope:

- The Dutch spectacle maker Zacharias Janssen (1585–1632) is credited with making one of the earliest compound microscopes (ones that used two lenses) around 1600. In the 1660s, another Dutchman, Antonie van Leeuwenhoek (1632–1723) made microscopes by grinding his own lenses. His simple microscopes were more like magnifying glasses, with only one lens.
- The compound microscope is so called because, in contrast to a single magnifying convex lens used in simple light microscope, it has two such lenses—the *objective* and the *eyepiece* to achieve higher magnification. The compound microscope can be *monocular* (has only one



FIGS. 1A AND B: (A) Monocular microscope; (B) Binocular microscope.

eyepiece) **(Fig. 1A)** or *binocular* (has two eyepieces to prevent eye strain) **(Fig. 1B)**.

PARTS OF THE MICROSCOPE

The Support System

The support system acts as a framework to which various functional units are attached **(Fig. 2)**:

- **Base:** It is a heavy metallic, U-shaped or horseshoe shaped base or foot, which supports the microscope on the worktable to provide maximum stability.
- **Pillars:** Two upright pillars project up from the base and are attached to the C-shaped handle. The hinge joint allows the microscope to be tilted at a suitable angle for comfortable viewing.

Note: The microscope is never tilted when counting cells in a chamber or when examining a blood film under oil immersion. It can be tilted for viewing histology slides.

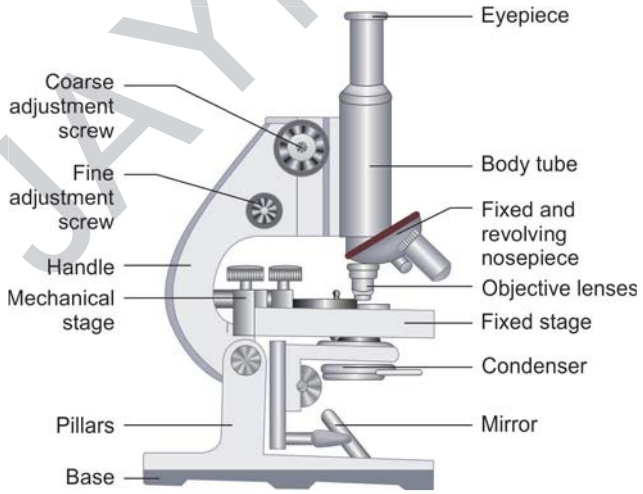


FIG. 2: Parts of the compound microscope (Monocular).

- **Handle (the arm or limb):** The curved handle, which projects up from the hinge joint supports the focusing and magnifying systems.
- **Body tube:** Fitted at the upper end of the handle, either vertically or at an angle, the body tube is the part through which light passes to the eyepiece, thus conducting the image to the eye of the observer. It is 16–17 cm in length, and can be raised or lowered by the focusing system.
- **The stage:** It has two components: the **fixed stage** and the **mechanical stage**.
  - **Fixed stage:** It is a square platform with an aperture in its center, and fitted to the handle (limb) below the objective lenses. The slide is placed on it and centered over the aperture for viewing. The converging cone of light emerging from the condenser passes through the slide and the objective into the body tube.
  - **Mechanical stage:** It is a calibrated metal frame fitted on the right edge of the fixed stage. There is a spring-mounted clip to hold the slide or counting chamber in position while two screw heads move it from side to side and forward and backward. The vernier scale on the frame indicates the degree of movement.

The Focusing System

- The focusing system consists of **coarse** and **fine** adjustment screw heads. It is employed for raising or lowering the optical system with reference to the slide under study till it comes into focus. Thus, the adjustments place an objective lens at its optimal working distance, i.e., its focal length.
- There are two coarse and two fine adjustment screws working on a double-sided micrometer mechanism, one pair (one coarse and one fine) on either side. If one coarse (or fine) adjustment is turned, its partner on the other side also rotates at the same time. It is, therefore, not sensible to use both hands on the coarse or the fine screws simultaneously.
- The coarse adjustment moves the optical system up or down rapidly through a large distance via a rack and pinion arrangement.
- The fine adjustment works in the same way but several rotations of the screwhead are required to move the tube through a small distance, e.g., one rotation moves the tube by 0.1 mm or less. The fine adjustment is usually graduated in 1/50th of 0.1 mm, where each division corresponds to a movement of 0.002 mm of the tube. It is employed for accurate focusing.

Note: The left hand is used both for coarse and for fine focusing, while the right hand is used for the mechanical stage to move the slide in various directions. The student is advised to get into the habit of using the hands simultaneously for different purposes.

The Optical (Magnifying) System

The optical system consists of the following:

- **The body tube:** The distance between the upper ends of the objectives and the eyepiece is called the tube length,

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which is 16–17 cm. The distance between the upper focal point of the eyepiece and the lower focal point of the objective is called **the optical tube length,** which is about 25 cm (A × 10 lens will produce an image 10 times the diameter of the object as it naturally appears when held at 25 cm from the eye).

- **The eyepiece:** The eyepiece fits into the top of the body tube. Most microscopes are provided with 5×, 8×, and 10× eyepieces, though 6× and 15× are also available. Each eyepiece has two lenses—one mounted at the top, the **"eye lens",** and the other, the **"field lens"** is fitted at the bottom. The field lens collects the divergent rays of the primary image and passes these to the eye lens, which further magnifies the image.
- **The nosepiece:** It is fitted at the lower end of the body tube and has two parts: the **fixed** nosepiece, and the **revolving** nosepiece. The latter carries interchangeable objective lenses. Any lens can be rotated into position when desired, its correct position being indicated by a "click".
- **Objective lenses:** Three spring-loaded objectives of varying magnifying powers are usually provided with the student microscope. In some cases, there is a place provided for a "scanning" lens as well. Each objective has a cover glass which forms its outer covering and protects it. Though each lens can be unscrewed for cleaning, the students are not supposed to remove them.

Note: "x" is the sign of multiplication. *The magnifying power of each objective, as that of the eyepiece, is etched on it.*

The objective lenses (Fig. 3) are:

- **Low power (LP) objective (10×):** The LP objective in common use magnifies 10 times. It is used for initial focusing and viewing a large area of the specimen slide. The numerical aperture (NA) of this lens is always less than that of the condenser in most microscopes. In order to achieve focus, therefore, the NA has got to be closely matched by reducing the light reaching the specimen under study. This is achieved by lowering the condenser to the lowest position and by slightly opening the iris diaphragm. LP objective has a yellow rim around its lower end.
- **High power (HP) objective (40×):** This lens magnifies the image 40 times. Because of its higher magnification, it is used for more detailed study of the material before switching to an oil immersion lens. The NA of HP lens is almost equal to, or slightly less than that of commonly used condenser. Therefore, the condenser has to be slightly raised midway and the iris diaphragm is also opened half to get more light and maximum clarity in focusing. This objective has a blue rim around it.
- **Oil immersion (OI) objective (100×):** The OI lens magnifies the image 100 times. Since the lens almost touches the slide, it has to be immersed in a special medium (most commonly cedar wood oil), a drop of which is first placed on the slide. The oil is used to increase the NA and thus the resolving power of the objective. Since the NA of the OI objective is always greater than that of the condenser. Thus the condenser has to be raised to its highest position and iris diaphragm should be fully opened. As this lens gives (with an eyepiece of 10×) a total magnification of 1,000 times, it is employed for detailed study of the morphology of blood cells and tissues **(Table 1)**. OI objective is marked by a black rim around its lower end.



FIG. 3: Objective lenses.

Table 1: Important features of compound microscope.

Objective lens	*Numerical aperture*	*Mirror (simple microscope)*	*Position of condenser*	*Iris diaphragm*
Low power (LP) (10x)	0.25	Concave	Lowest position	Slightly open
High power (HP) (40x)	0.65	Concave	Midway	Half open
Oil immersion (OI) (100x)	1.25	Plane	Highest position	Fully open

Parfocal system: The objectives these days are so constructed that when one lens (LP, for example) is in focus, the others are more or less in focus. Thus, switching from one lens to another (e.g., from LP to HP) requires only a little turn of fine adjustment to bring the image into sharp focus. This arrangement of lenses is called "parfocal system."

The Illumination System

No microscope can function optimally unless proper illumination (lighting) is provided. All the light that will reach the eye should come from the specimen under study. Light from any other part of the slide will tend to obscure the details. Such extra (extraneous) light is called glare. The illumination system must, therefore, provide uniform, soft, and bright illumination of the entire field of view. Two factors are involved in providing such uniform illumination:

1. The construction and position of the condenser.
2. The size of the iris diaphragm.

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The clarity of an image depends on an optimal (ideal) amount of light available. The illumination (the process of providing light) can be altered by raising or lowering the condenser and opening or closing the diaphragm. A proper combination of the two has to be selected under different conditions. In general, we require less illumination when viewing a clear, unstained object, and greater illumination when viewing a stained preparation.

The illumination system of the bright-field microscope consists of: a source of light, and a mechanism to condense the light and direct it into the specimen under study.

- **Source of light:** The light source may be outside the microscope or within the microscope.
  - **External light source:** It may be the diffuse, natural daylight (sunlight) reflected and scattered by the atmosphere and its dust particles and reflected from the buildings. On bright, sunny days, the north daylight, which is a distant light source, is ideal for routine student work.
    If daylight is not available, or is not sufficient, an artificial source of light—a fluorescent tube, or an electric lamp housed in a lamp box with a frosted glass window, fitted on the worktable can provide enough light.
  - **Internal light source:** In most microscopes, there is a provision to remove the mirror and fit an electric microscope lamp in its place. This unit has frosted tungsten lamp to provide uniform white light.
- **The mirror:** A double-sided mirror, in fact two mirrors, one flat or plane and the other concave, fitted back to back in a metal frame is located below the condenser; it can be rotated in all directions.
  - The plane mirror is used with a distant source of light (natural, or daylight). The parallel rays of light are reflected parallel into the condenser.
  - The concave mirror, on the other hand, is employed when the light source is near the microscope. The divergent rays of light are reflected as parallel rays and directed into the condenser.
- **The condenser ("substage" or "substage condenser"):** The condenser is a system of lenses fitted in a short cylinder that is mounted below the stage. It can be raised or lowered by a rack and pinion, and concentrates the light rays into a solid cone of light that illuminates the material under study. It also helps in resolving the image.
  - **The lens system:** The commonly used substage is *Abbe-type condenser*. It is composed of two lenses which should be corrected for spherical and chromatic aberrations.
    Since the condenser is a lens system, it has a fixed NA, which should be equal or less than that of the objective being used. Raising or lowering the condenser can vary its NA. And with the axes of the two being the same, all the light passing through the condenser is collected by the objective, thus allowing maximum clarity.
  - **The iris diaphragm:** It is fitted within the condenser. A small lever on the side can adjust the size of the aperture of the diaphragm, thus allowing more or less light falling on the material under study. Reducing the size of the field of view (i.e., by narrowing the aperture) decreases the NA of the condenser. Thus, proper illumination includes a combination of position of light source, regulation of light intensity, position of condenser, and regulation of the size of field of view.
  - **Filter:** A metal ring can accommodate a pale blue or green filter since monochromatic light is ideal for microscopy.

Generally, when viewing clear preparations under low power, we need less light, but more illumination is required when studying stained preparations under oil immersion lens.

PHYSICAL BASIS OF MICROSCOPY

Resolving Power (Resolution)

- The utility of a microscope depends not only on its magnifying power but also on its power of resolution, i.e., its ability to show closely located structures as separate and distinct from each other. This translates into the ability to improve the details of structures within a cell.
- The resolving power of a lens depends on its NA as well as the wavelength of light. With the light microscope and OI lens of 100×, and NA of 1.30, its resolving power is about 0.25 μm (2,500 Angstroms) with white light, and about 0.19 μm with monochromatic green light (shortest wavelength: 5.5×10^{-5} cm). The electron microscope, however, gives very high magnifications and can separate dots that are about 0.5 nm apart or even less.

Note: 1 nanometer (nm) = 0.001 micrometer (μm) = 0.000001 mm.

- The resolving power of a microscope is expressed in terms of ***limit of resolution (LR),*** or the minimum separable distance. If this distance is less than LR, the two points appear as one. The formula for determining LR is: LR = 0.61 × W/NA, where W = wavelength of light being used, and NA = the numerical aperture of the objective in use.

Magnification

In order to see clearly and distinctly, the details and contours of closely-located structures (say in a cell), and their image has to be magnified many times. How this is achieved is explained below.

Calculation of Total Magnification

Since the objective and eyepiece both magnify the image, it is easy to calculate the total magnification of any combination of objective lens and eyepiece. For example, with an eyepiece of 10×, the magnifications with the three objectives will be:

Low power objective (10×) = 10 × 10 = 100 times.
High power objective (40×) = 40 × 10 = 400 times.
Oil immersion objective (100×) = 100 × 10 = 1000 times.

Numerical Aperture

- The numerical aperture of a microscope objective is the measure of its ability to gather light and to resolve fine specimen detail while working at a fixed object (or specimen) distance. In simple terms, numerical aperture

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(NA) is a number that measures how well a microscope objective can gather light and resolve fine details.

- As light passes through a specimen, it enters the objective as an inverted cone. The maximum angle of the cone of light rays collected by the front lens of the objective is known as the 'angular aperture'. While the angular aperture is directly proportional to the lens diameter, it is also inversely proportional to the focal length of the lens. As focal length decreases, there is an increase in the amount of light which the objective front lens can gather. In other words, if the objective is very close to the specimen, then more oblique light rays can be collected by the objective lens. A lens with a larger diameter can capture more light from a wider range of angles, resulting in a larger angular aperture. A wider angular aperture allows for better light gathering, which can improve image quality in low-light conditions.
- The numerical aperture can be expressed and determined by the following formula: **Numerical aperture (NA) = n • sin(α)**—where 'n' is the refractive index of the medium between the cover slip/glass slide and the lens of the objective (for example; air, water or oil). The **'α'** symbol or 'alpha' is the half of the angle of the cone of light which can be collected by the objective lens, as shown in **Figure 4**. Higher NA values allow more oblique rays to enter the lens, which produces a higher-resolution image. This, NA of a lens is an index of its power of resolution.
- As explained above, NA is also an index of light gathering power of a lens, i.e., the amount of light entering the objective. The NA can be decreased by decreasing the amount of light passing through the lens. Thus, as shown below, the illumination has to increase as the objectives are changed from LP to HP to OI. The magnifying power of each lens and its NA rather than its focal length, are etched on each objective lens.
- **Low power objective (10×; NA = 0.25; focal length = 16 mm)**
- **High power objective (40×; NA = 0.65; focal length = 4 mm)**
- **Oil immersion objective (100×; NA = 1.30; focal length = 2 mm).**

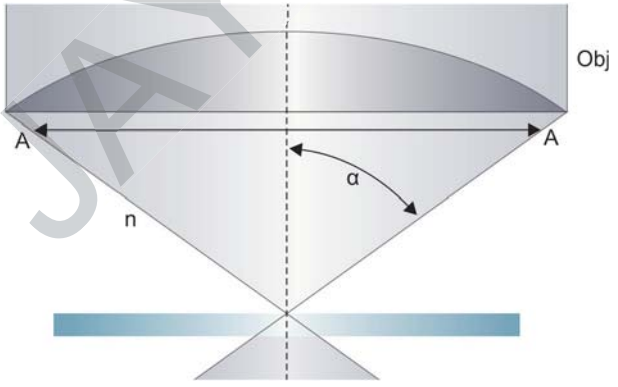

FIG. 4: Diagram to explain the numerical aperture. The angular aperture (A) is the maximum angle of the light cone collected by the objective lens. The angle alpha (α) is half of the angle of the light cone. 'n' is the refractive index of the medium in between the specimen and objective lens.

Image Formation in the Compound Microscope

- It is the objective that starts the process of magnification. It forms a real, inverted, and enlarged image (primary image: A'-B') **(Fig. 5)** in the upper part of the body tube (A real image is that which can be received on screen).
- The field lens of the eyepiece collects the divergent rays of light of the primary image and passes these through the eye lens, which therefore the image seen by the eye is—virtual, inverted, and magnified, and appears to be further magnified the image. The light rays reaching the observer's eye are divergent and about 25 cm in front of the eye. **Figure 5** shows the ray diagram of a compound microscope.

Working Distance

The working distance is the distance between the objective and the slide under study. This distance decreases with increasing magnification. It is 8–13 mm in LP, 1–3 mm in HP, and 0.5–1.5 mm in OI lenses, respectively. **Figure 6** shows the approximate working distances for each lens. Note that the OI lens has to be immersed in a drop of oil.

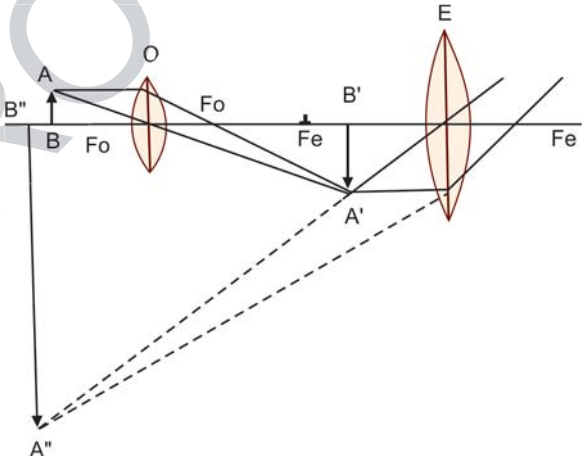

FIG. 5: The ray diagram of a compound microscope. AB = object; A' B' = real, inverted, magnified image; A" B" = virtual, inverted, magnified image; O = objective lens; E = eyepiece; Fo = focus of objective; Fe = focus of eyepiece.

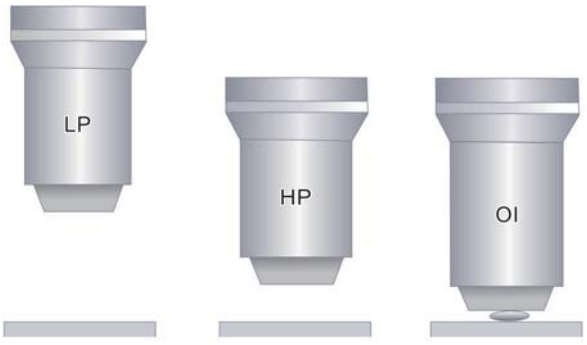

FIG. 6: Diagram to show the working distances of low power (LP), high power (HP) and oil immersion (OI) lenses.

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PROCEDURE

Principle

A focused beam of light passes through the material under study into the microscope. Parts of the specimen that are optically dense and having a high refractive index or are colored with a stain (dye), cast a potential shadow which is magnified in two main stages as it passes into the observer's eye.

Important: Students using glasses should put on their glasses while using the microscope. A proper illumination of the specimen slide is very essential. The broad rule about illumination is described in **Table 2.** The slide is first viewed under low magnification to get a general view all over. One can then choose an area of interest for viewing it under higher magnifications. First use the coarse adjustment screw to focus the slide till the object has become visible. Then use the fine adjustment to obtain the exact focus.

Focusing Under Low Power

- Place the microscope on your worktable in an upright position, and raise the body tube 7–8 cm above the stage. Put the slide on the stage and, using the mechanical stage, bring the specimen over the central aperture.
- Select and adjust the mirror (plane or concave) so that the light shines on the specimen.
- **Under low power before focusing the object, the condenser is brought to the lowest position and the iris diaphragm is slightly opened to cut down excess light.** The position of the condenser is put to the lowest with the LP lens because the aperture of the LP lens is wide, a high condenser would allow too much light to enter the microscope and cause glare.
- Looking from the side, and using the coarse adjustment, bring the body tube down so that the LP lens is about 1 cm above the slide. Now look into the eyepiece and gently raise the tube till the specimen comes into focus. But if it does not, i.e., if you have missed the focusing position, repeat the whole procedure. When the image comes into focus, scan the entire field, racking the fine adjustment all the time.

Focusing Under High Power

- For focusing under high magnification, simply rotate the nosepiece so that the HP lens clicks into position.
- **The condenser has to be slightly raised midway and the iris diaphragm is also opened half to get more light and maximum clarity in focusing: Use fine adjustment as required.**
- If the lens system is not parfocal, look from the side and bring the lens down to about 1–2 mm above the slide. Now look into the microscope and raise the tube slowly and gently till the image comes into focus.

Table 2: Characterization of illumination.

Objective	*Condenser position*	*Iris diaphragm*
Low power (10x)	Low	Partly open
High power (40x)	Midway	Half open
Oil immersion (100x)	High	Fully open

Focusing Under Oil Immersion

This objective is the most frequently used in hematology because of its high magnification and resolution (It can also be used for mounted histology and pathology slides). *The two features of this objective are its very small aperture (pinhole sized aperture) through which light enters it, and its deep focusing position that is about 1 mm from the slide.* The small pinhole sized aperture allows only the central cone of light to pass through and form the image. Had the diameter been large, excessive refraction would have caused spherical and chromatic aberrations, thus making the image indistinct.

- **The condenser has to be raised to its highest position and iris diaphragm should be fully opened.** The highest position of the condenser with the oil immersion lens allows enough light to enter it through its pin-hole aperture.
- Oil immersion objective is immersed in oil: There is a thin layer of air between this objective and the glass slide when the lens is in focus (without the oil the image can be seen but it is very faint and blurred). When light passes from a denser medium (glass of the slide) into a rarer medium (the thin layer of air), they are refracted away from the normal. As a result, when light rays emerge from the slide, many of them are refracted away from the aperture of the objective and very few enter it, and a faint image results. Cedar wood oil, which has the same refractive index as that of glass, i.e., 1.55 (air = 1.00; water = 1.33), removes this layer of air so that the glass of the slide and the objective lens become a continuous column (thus avoiding refraction) and allow enough light to enter the objective to provide a clear image. **Other mediums that can be used are glycerin and paraffin, their refractive index being 1.35–1.40. However, cedar wood oil, though costly, gives best results.**
- Raise the body tube so that the OI lens is about 8–10 cm above the slide. Place a drop of cedar wood oil on the slide, and looking from the side, slowly bring the objective down till it just enters the oil drop. The oil will spread out in the capillary space between the slide and the lens (thus effectively removing the thin layer of air).
- While looking into the eyepiece, slowly and very carefully raise the objective with coarse adjustment (without taking it out of the oil) till the cells come into view (if no cells are seen, repeat the whole process). Use the fine adjustment for fine tuning. When you move the slide, the oil will move with it. It is therefore a bad habit to cover the entire slide with oil to begin with.

"Racking the Microscope"

The cells and their constituents are three-dimensional structures and lie at different levels. Therefore, it is important not to keep a fixed focus but **to continuously "rack" the microscope** by using fine adjustment after the specimen has been brought under focus under any magnification. By turning the fine adjustment screw this way and that, various structures come into and go out of focus alternately.

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COMMON DIFFICULTIES ENCOUNTERED BY STUDENTS

Some common problems encountered are:

- **The material cannot be focused or the image is very faint:**
  - The slide may not be near the focus of the objective, or there may be no visible material under it (e.g., part of the blood film may be missing from this area). Check this out and start with coarse adjustment once again.
  - The slide bearing the material may have been placed upside down on the stage, a common mistake made by the students with a blood film. The thickness of the glass slide does not allow the OI lens to reach down to its working distance. Reversing the slide will solve the problem.
  - If focusing is achieved with LP and HP lenses but not with OI lens despite all efforts, the lens may have been damaged earlier. Seek the help of your tutor.
- **There may be a dark shadow in the field:** If the shadow rotates when the eyepiece is rotated, remove it and clean it. Or there may be an air bubble in the cedar wood oil.
- **The field of view appears oval instead of round:** This problem arises when the objective has not been properly "clicked" into position.
- **The illumination of the image is poor:** Check the source of light, angle of the mirror, the position of the condenser, and iris diaphragm.
- **The image does not come into focus even when the objective is in the lowest position and the fine adjustment cannot move down any further:** This happens when the fine adjustment screw reaches the end of its thread (turn) before the image is brought to its focus. To overcome this problem, turn the adjustment screw in the opposite direction for several turns and then use the coarse adjustment screw to regain the focus once again (it is, therefore, best to keep the fine adjustment screw near the middle of its turning range).

PRECAUTIONS

1. Select a stool or chair of suitable height so that your eyes are at a level slightly above the eyepiece. This will ensure comfortable working for long periods.
2. Ensure that all the lenses are clean and free from dust and smudges. Do not touch them with your fingers, nor blow on them to remove dust.
3. Check the position of the objective, condenser, and diaphragm, to ensure optimal illumination.
4. Never lower any objective from any height while looking into the microscope otherwise one might miss the focusing position and moving it down continuously might break the slide or scratch the objective lens permanently.
5. Once a specimen has been focused, continuously "rack" the microscope.
6. Cleaning the microscope. Never leave cedar wood oil on the OI lens, because it may seep into the body of the objective and damage the lens permanently. Dried oil is difficult to remove. Remove oil with lens paper and then use xylene to clean the lens.
7. Cover the microscope with the plastic cover after use.

OTHER TYPES OF MICROSCOPES

Some of these are:

1. **Dissection microscope:** It is a binocular microscope used for micro dissection under magnification.
2. **Dark-field microscope:** It employs a special condenser that causes light waves to cross on the material under study rather than passing through it. As a result, the field of view appears dark (hence called "dark-field" in contrast to "bright-field" microscopy) against which the object appears bright. It is used in microbiology to study spirochetes.
3. **Phase-contrast microscope:** Since the living cells are mostly transparent, they must be stained with vital stains, or they must be first fixed in alcohol and then stained with acid or basic dyes before they can be viewed under the microscope. In this microscope, a special phase plate is inserted into the condenser, which can retard the speed of some light waves. Since the tissue cells and organisms have different refractive indices, this microscope uses these differences to produce an image with good contrast of light and shade. Thus, unstained wet preparations can be studied (e.g., platelets). The interference microscope is based on similar principle.
4. **Interference-contrast microscope:** A special prism that can split a beam of light is added to the condenser. The two split beams are then polarized, but only one resultant beam passes through the specimen under study while the other (reference beam) does not. The two beams are then recombined to produce a three-dimensional image.
5. **Polarizing microscope:** It has a polarizer (filter), which is usually placed between the light source and the specimen, and an analyzer, which is located between the objective and the eyepiece. Such a system is used to study tissues that have the property of birefringence (e.g., muscle fibers).
6. **Fluorescence microscope:** A fluorescent dye is used to stain tissues which are then studied under this microscope.
7. **Transmission electron microscope (TEM):** Invented by Knoll and Ruska in 1940, the TEM uses a strong beam of electrons instead of light and electromagnetic fields in place of glass lenses. The electrons produce a wavelength of about 0.05 Å, and provide a practical resolution of about 5 Å (theoretically possible resolution is about 1 Å). The magnified image, which is visible on a fluorescent screen, can be recorded on a photographic film, and the negative further enlarged 6–8 times. Thus, the total magnification obtained can vary from one to several hundred thousand times.
8. **Scanning electron microscope (SEM):** This microscope, which achieves a resolution of about 30 Å, has been developed for three-dimensional study of surface

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topography of cells and object. Though similar to TEM, the SEM employs a different technique.

OBJECTIVE STRUCTURED PRACTICAL EXAMINATION

Aim: To focus a given slide of blood film under LP/HP/OI lens.
Procedural steps: See text above.
Checklist:

1. Raise the body tube, put the slide on the stage, and use mechanical stage to bring the object over the central aperture.
2. Choose the light source and correctly bring the objective lens into position:
   i. LP ii. HP iii. OI
3. Adjust the position of the condenser and iris diaphragm for:
   i. LP ii. HP iii. OI
4. Look from the side while lowering the body tube.
5. Adjust the light and use coarse and fine adjustment screws.
6. Rack the microscope constantly.

QUESTIONS

1. **What is a compound microscope? What type of image is produced by it?**
2. **When is a plane mirror used and when concave?**
3. **What is the total magnification and how will you calculate?**
4. **What is meant by the term numerical aperture? What is its significance?**
5. **a. How will you identify an oil-immersion objective lens?**
   b. Why is cedar-wood oil used with this lens and not with others?
6. **Will you see any image with the oil immersion lens without the cedar wood oil?**
7. **Why does the oil immersion objective have a pinhole sized aperture?**
8. **Why should the position of the condenser be low with the low power (LP) lens and highest with oil immersion lens?**
9. **Why are different degrees of illumination required when using a microscope and why?**
10. **What is meant by racking the microscope and what is its importance?**
11. **What are the other types of microscopes?**

1.2: EXPERIMENTS ON BLOOD

STUDENT OBJECTIVES

After completing this experiment, the student should be able to:

- Explain what is a blood sample, what are its sources and what are its main constituents?
- Describe the purpose of collecting a blood sample.
- Indicate how to attain and maintain asepsis when collecting a blood sample.
- Collect capillary blood from a finger prick, heel-prick, and earlobe prick, and precautions to be taken during a skin-prick.
- Indicate the steps for obtaining a blood sample by venipuncture.
- Name the various anticoagulants employed in hematological studies and their mode of action.
- Provide samples of plasma and serum.

INTRODUCTION

There are two common sources of blood for routine laboratory tests: ***blood from a superficial vein*** or ***from skin capillaries.*** In addition to these two arterial blood may be required for special tests.

Asepsis

The term asepsis refers to the condition of being free from septic or infectious material—bacteria, viruses, etc. The skin is a formidable barrier to the entry of foreign invaders and the first line of defense against bacteria and other disease-causing microorganisms which are present in abundance on the skin and in the air. Therefore, puncturing the skin always poses the danger of infection. In order to achieve asepsis, the following aspects need to be kept in mind.

Sterilization of Equipment

All the instruments to be used for collecting blood—syringes, needles, lancets, and cotton and gauze swabs—should preferably be sterilized in an autoclave. The old practice of boiling glass syringes and needles in tap water is now obsolete. Irradiated and sealed, single-use syringes, needles, lancets and blades are now freely available and are in common use.

Cleaning/Sterilization of Skin

Every aseptic precaution must be exercised to clean and sterilize the selected area. The area, after washing, should be allowed to dry before applying the antiseptics because these agents do not act well on wet skin. At least 2–3 sterile cotton/gauze swabs soaked in 70% alcohol, methylated spirit, or ether should be used to clean and scrub the area. Cotton swabs are likely to leave fibers sticking to the skin and provide an undesirable contact, or they may appear as artifacts in a blood film. But if they are used, the final cleaning should be done with a gauze swab.

Note: After cleaning the skin, allow the alcohol to dry by evaporation (do not blow on it), because sterilization with alcohol is effective only after it has dried.

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Prevention of Contamination

Any material used for skin puncture, or the operator's hands may cause contamination. Therefore, once the site has been cleaned and dried, it should not be touched again.

Physical Characteristics of Blood

- Blood is a complex connective tissue in which living blood cells and the formed elements are suspended, which transports substances from one part of the body to another.
- Blood volume refers to the total amount of fluid circulating within the arteries, capillaries, veins, venules, and chambers of the heart at any time. The total blood volume is around 5–6 L, i.e, 8% of body weight (assuming 70 kg as average body weight of a normal adult).
- The various physical characteristics are as follows:
  - *Color*: Depending on the amount of oxygen it is carrying, the color of blood varies from scarlet (oxygen-rich) to a dull red (oxygen-poor).
  - *Viscosity*: Blood is about five times thicker or more viscous than water, largely because of its formed elements.
  - *Specific gravity:* 3.5–5.4 times that of water.
  - *pH*: Blood is slightly alkaline, with a pH between 7.35 and 7.45.

Composition of Blood

Blood consists of:

- **Formed elements which account for 45%:** These include red blood cells (erythrocytes), white blood cells (leukocytes) and platelets.
- **Plasma makes up 55%:** The fluid portion of blood is called plasma, which contains different types of proteins and other soluble molecules. It is approximately 91% water and 9% solids. It is the clear straw colored portion of the blood and represents 5% of body weight.

COLLECTION OF BLOOD SAMPLE

The term "blood sample" refers to the small amount of blood—a few drops or a few milliliters—obtained from a person for the purpose of testing or investigations. These tests are carried out for aiding in diagnosis and/or prognosis of the disease or disorder.

Selection of Site for Skin Prick

Sources and Amount of Blood Sample

1. **Capillary blood:** The skin and other tissues are richly supplied with capillaries, so when a drop or a few drops of blood are required, as for estimation of Hb, cell counts, bleeding time (BT) and CT, blood films, microchemical tests, etc., blood from a skin puncture (skin-prick) with a lancet or needle is adequate. Capillary blood is also called **"peripheral blood"** as it comes out of the peripheral vessels (capillaries) in contrast to venous blood **(Table 3)**. In adults and older children, capillary blood is generally obtained from a skin puncture made on the tip of the middle or ring finger, or on the lobe of the ear. In infants and young children in whom the fingers are too small for a prick, the medial or lateral side of the pad of the big toe or heel is used. The site for skin-prick should be clean and free from edema, infection, skin disease, callus, or circulatory defects.
2. **Venous blood:** When larger amounts (say, a few milliliters that cannot be obtained from a skin puncture) are needed for complete hematological and biochemical investigations, venous blood is obtained with a syringe and needle by puncturing a superficial vein. In infants, venous blood may have to be taken from the femoral vein, or the frontal venous sinus.
3. **Arterial blood:** When arterial blood is needed for special tests such as blood pH, gas levels, etc., an artery such as radial or femoral is punctured with a syringe and needle. This, however, is not a routine procedure.
4. **Cardiac catheterization:** Blood from a heart chamber, taken through a cardiac catheter, may be required for special tests. Such local sampling offers a unique way of assessing the local cardiac milieu, this may prove useful in the monitoring of both local/systemic drug therapies and interventional technologies.

Table 3: Differences between venous and capillary blood.

Venous blood	*Capillary blood*
It is obtained from a superficial vein by venipuncture	It is obtained from a skin puncture, usually over a finger, ear lobe/or the heel of a foot
A clean venipuncture provides blood without any contamination with tissue fluid	Blood from a skin prick comes from punctured capillaries and from smallest arterioles and venules
There is less risk of contamination since sterile syringe and needle are used	There is greater risk of contamination and transmission of disease as one may be careless about sterilization since skin prick is considered a harmless procedure
Cell counts, Hb, and PCV values are generally higher	These values are likely to be on the lower side since some tissue fluid is bound to dilute the blood even when it is free-flowing
Venous blood is preferable when normal blood standards are to be established, or when two samples from the same person are to be compared at different times	Capillary blood is not suitable for these purposes

Containers for Blood Sample

A container is a receptacle into which blood is transferred from the syringe before sending it to the laboratory. Clean and dry glass test tubes and collection bottles are the usual ones to be used. A container may or may not contain an anticoagulant depending on whether a sample of blood/plasma, or serum is required.

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- **For a sample of whole blood or plasma:** The blood is transferred to a container containing a suitable anticoagulant. This is to prevent clotting of blood.
- **For a sample of serum:** No anticoagulant is used. The blood is allowed to clot in the container and serum is collected.

Collection of Blood Samples

Collection of Capillary Blood (Skin-prick Method)

A single bold prick is given on the finger under aseptic conditions **(Fig. 7)**. **Never squeeze the pricked finger** as this will expel tissue fluid along with blood to come out of the puncture site. The dilution of blood will, thus, nullify the results. *Hence for clinical work, venous blood is always preferred.* Skin-prick may be used on the bedside of a patient, or in an emergency when it is not convenient to take a venous sample.

Note: The thumb and little finger are never pricked because the underlying palmar fascia (venous bursae) from these digits are continuous with those of the forearms. Any accidental injury to these fasciae may cause the infection to spread into the forearm. Thus, finger prick is given on the distal digit on the palmar surface of the 3rd/4th finger.

Remember that one deep puncture, which will give you free flowing blood, is less painful than 3 or 4 superficial pricks.

Apparatus

- **Blood lancet/pricking needle:** Disposable, sterile, one-time use, blood lancets (flat, thin metal pieces with 3–4 mm deep penetrating sharp points) are commercially available and should be preferred.
  Ordinary, narrow-bore **injection needles** are useless since they only make shallow cuts rather than deep punctures. However, wide-bore (22 gauge) needles may be used in an emergency or if blood lancets are not available.
  Cutting needles with three-sided cutting points (used by surgeons) can serve the purpose well.
  Pricking gun: A spring-loaded pricking gun that has a disposable, three-sided sharp point, and a loading and releasing mechanism, is ideal because the depth of the puncture can be preselected.
- Sterile gauze/cotton, moist with 70% alcohol/methylated spirit.
- Glass slides, pipettes, etc., according to requirements.



FIG. 7: Finger prick method.

Note: The students should bring their own lancets. Spirit does not kill the hepatitis virus which can be killed on heating. However, too much heating, however, is likely to blunt the pricking points.

Procedure

All aseptic precautions must be taken. The person giving the prick should wash his/her hands with soap and water, and wear gloves if possible.

- Clean and vigorously rub the ball of the finger with the spirit swab, followed by a final cleaning with dry gauze (scrubbing increases local blood flow).
- Allow the alcohol to dry by evaporation for the following reasons:
  - Sterilization with alcohol/spirit is effective only after it has dried by evaporation.
  - The thin film of alcohol can cause the blood drop to spread sideways along with alcohol so that it will not form a satisfactory round drop.
  - The alcohol may cause hemolysis of blood.
- Steadying the finger to be pricked in your left hand, apply a gentle pressure on the sides of the ball of the finger with your thumb and forefinger to raise a thick, broad ridge of skin (do not touch the pricking area).
- Hold the lancet between the thumb and fingers of your right hand, and keeping it directed along the axis of the finger, but slightly "off" center so as to miss the tip of the phalanx, prick the skin with a sharp and quick vertical stab to a depth of 3–4 mm and release the pressure. The blood should start to flow slowly, spontaneously and freely (without any squeezing)—if a good prick has been given.

Important: Do not squeeze or press the finger as the tissue fluid squeezed out will dilute the blood and give false low values. The squeeze also tends to close the wound edges. You may exert a slight tension on either side of the puncture with your thumbs in order to open up the wound more widely (a plug of epithelial cells tends to block the puncture especially if the wound is shallow, as often happens if a narrow-bore injection needle is used). The forearm or the hand may be squeezed or milked toward the fingers to facilitate blood flow. If all efforts fail, a fresh prick may be required.

- Wipe away the first two drops of blood with dry, sterile gauze as it may be contaminated not only with tissue fluid, but also with epithelial and endothelial cells which will appear as artifacts in the blood film.
- Allow a fresh drop of blood of sufficiently large size (about 3–4 mm diameter) to well up from the wound, and make a blood smear, or fill a pipette as the case may be.
- Clean the area of the prick with a fresh swab and ask the subject to keep the swab pressed on the wound with his/her thumb till the bleeding stops, which occurs in a minute or so.

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Earlobe Prick

With the use of a sterile needle or corner edge of a sterile blade give a 2-mm deep prick (the skin here is usually thinner than at the fingertip). Wipe away the first drop and allow a new one to form).

Note: The BT and CT tests give better results here than at the finger prick.

Pricking the Heel (Fig. 8A)

In infants and young children, blood can be collected from the cleaned and warmed medial or lateral areas of the heel.

Note: The central plantar and the posterior curvature areas of the heel should be avoided as the prick may cause injury to the underlying tarsal bones which lie near the surface.

Precautions

1. Keep the equipment for the test ready before getting/giving a finger prick.
2. The selected site should be clean, free from infection, edema, or skin disease.
3. The site should be vigorously cleaned and scrubbed with sterile gauze and alcohol. Scrubbing increases local blood flow.
4. The lancet/needle should be sterile, and if it is to be reused, it should be passed through a flame.
5. The puncture should be deep enough to give free flowing blood but not so very deep that it takes inordinately long time for the bleeding to stop.
6. Do not press or squeeze the finger to increase the blood flow from the skin-prick



FIG. 8A: Sites of heel puncture.

Collection of Venous Blood

The median cubital vein in the forearm is usually chosen for venipuncture **(Figs. 8B and C)**. The blood sample from a vein must be collected by a medically qualified staff member who should screen the volunteer for any communicable diseases/especially viral hepatitis, and AIDS. Do not touch blood other than your own. Puncturing a vein and withdrawing blood from it needs assistance and complete aseptic precautions.

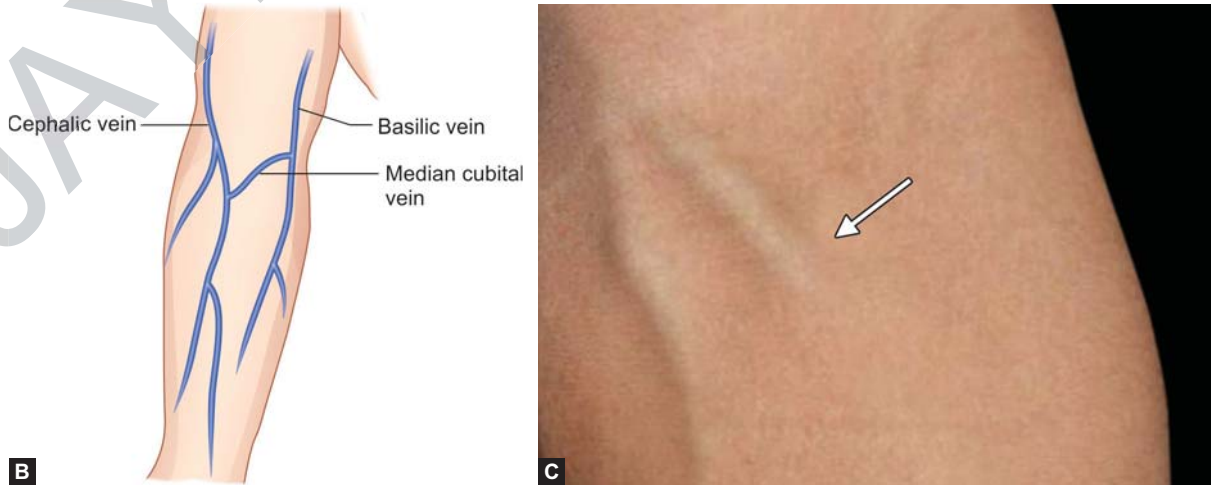
The following samples are not suitable for hematological tests:

1. *Clotted samples*: Even tiny clots in the anticoagulated blood can negate the results.
2. *Hemolyzed samples*: The red cells may be damaged and ruptured during collection or handling of blood. The released Hb tinges the plasma or serum red, rendering the sample unfit for tests.

For a sample of whole blood or plasma: (Plasma = Blood minus all the blood cells). Draw blood from a vein as and transfer it from the syringe to a container containing a suitable anticoagulant. Mix the contents well without frothing. A sample of whole blood is now ready for tests.

If plasma is desired, centrifuge the anticoagulated blood for 20–30 minutes at 2,500 rpm. Collect the supernatant plasma with a pipette and transfer it to another container (The packed RBCs will be left behind).

For a sample of serum: (Serum = Plasma minus fibrinogen and all the clotting factors). Transfer the blood from the syringe to a container *without any anticoagulant* in it, and



FIGS. 8B AND C: (B) Forearm veins, (C) Median cubital vein for venipuncture.

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keep it undisturbed. After the blood has clotted in an hour or two and the clot shrunk in size, the serum will be expressed. Remove the supernatant serum with a pipette and transfer it to a centrifuge tube. Centrifuge it to remove whatever red cells may be present. Clear serum can now be collected with another pipette.

Apparatus

Keep the following equipment ready before venipuncture:

1. Disposable gloves.
2. Sterile, disposable, one-time use, 10 mL syringe with side nozzle.
3. 10 mL test tubes/vials with or without anticoagulant.
4. Sterile gauze pieces moist with 70% alcohol/methylated spirit.
5. Tourniquet: A 2–3 cm wide elastic bandage with Velcro strips to keep it securely in place can be used. An inflated BP cuff can also be used.

Procedure

- Seat the subject comfortably on a chair with an arm rest.
- Compress the upper arm with his hands to make the veins prominent. The antecubital (medial basilic) vein is embedded in subcutaneous fat and is usually sufficiently large to take a wide-bore needle. It also runs straight for about 3 cm, and is usually palpable—even in obese subjects. If the vein is neither visible nor palpable, try the other arm. (You should avoid superficial veins because they are notoriously slippery. Veins above the ankle or on the back of the hand may have to be used).
- Once a suitable vein has been selected, support the subject's arm over the edge of the table. Wash your hands with soap and water, dry them on a sterile towel, and put on gloves. Take out the syringe and attach the needle (it is attached/detached with a little twist), with its bevel facing you.
- Apply the tourniquet about 2–3 cm above the elbow to obstruct the venous return. The subject may open and close her fist to increase the venous return and make the veins engorged (filled) with blood. If the vein is still not sufficiently prominent, a few "slaps" with your fingers over the region may do so.
- Clean the skin over the selected vein with gauze and alcohol and allow it to dry.
- Hold the syringe with the plunger pushed in, between your fingers and thumb of the right hand.
- With the first finger placed near the butt of the needle, puncture the skin and push in the needle under the skin with a firm and smooth thrust to the skin. Insert the needle proximally (i.e., in the direction of venous blood flow), with the bevel facing up, along the midline of the vein at a shallow angle (about 10–30°) to the skin.
- Slightly pull the plunger back with your thumb and little finger to produce a little negative pressure in the syringe. Advance the needle gently along the vein and puncture it from the side, a few mm ahead of the skin puncture. This prevents counter-puncture of the far wall of the vein and formation of a hematoma (local leakage of blood).
- As the vein is punctured, all resistance will suddenly cease and blood will start to enter the syringe. Do not withdraw blood faster than the punctured veins are filling; as too much pressure applied to the plunger is likely to cause mechanical injury and hemolysis of red cells. The subject may open and close the fist to enhance venous return.
- When enough blood has been collected, release the tourniquet and press a fresh swab over the skin puncture. Withdraw the needle gently but keep the swab in position. Ask the subject to flex the arm and keep it so to maintain pressure on the puncture site till the bleeding stops.
- Expel the blood gently into the container; do not apply force as it may cause mechanical injury to red cells. Gently shake, or swirl the container between your palms so that the anticoagulant (if used) mixes well with the blood without frothing.

Important: Try to access the vein efficiently and collect the blood sample within 30 seconds after tourniquet placement. Do not leave the tourniquet on for >1 minute. This is because stagnation of blood in the vein is likely to alter its composition—the cell counts usually increasing.

Precautions

1. All aseptic precautions must be observed and disposable gloves, syringe and needles must be used. The syringe, needle and the container should be clean and dry. The needle should be of wide bore, and the blood should be drawn slowly and without frothing. The needle should be removed from the syringe and blood should be expelled slowly into the container.
2. The tourniquet (or the BP cuff) must be removed before taking the needle out of the vein to avoid formation of hematoma.
3. The blood from the syringe should be transferred to the container without delay to prevent clotting.
4. Ask the subject to keep the swab in position till the bleeding from the puncture site stops.

COMMONLY USED ANTICOAGULANTS

Anticoagulants, also known as blood thinners, are chemical substances that prevent or reduce coagulation of blood, thus prolonging the clotting time.

Anticoagulants for In Vitro Use

1. **Ethylene diamine tetraacetic acid (EDTA):**
   - Both the potassium and sodium salts of EDTA are strong anticoagulants. The dry (anhydrous) dipotassium salt of EDTA, being more readily soluble than the sodium salt, is the anticoagulant of choice. The tripotassium salt of EDTA causes some shrinkage of RBCs that results in 2–3% decrease in packed cell volume.
   - **Mode of action:** EDTA prevents clotting by removing ionic calcium (which is an essential clotting factor) from the blood sample by **chelation**. The platelets appear clear and are neither aggregated nor destroyed.

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- **Preparation:** EDTA is used in a concentration of 1 mg/mL of blood. 0.2 mL of 2.5% solution of the salt placed in a container and dried in gentle heat in an oven is sufficient for 5 mL of blood. This provides 1 mg of EDTA/mL of blood (a number of containers can be prepared from the stock solution at a time).
- **Uses:** Except for coagulation studies EDTA is used for most hematological tests.

Note: Excess of EDTA (more than 2 mg/mL blood) affects all blood cells. Red cells shrink, thus reducing PCV, while WBCs show degenerative changes. Platelets break up into large enough fragments to be counted as normal platelets. Care should, therefore, be taken to use the correct amount of EDTA, and blood should be thoroughly mixed with the anticoagulant.

2. **Trisodium citrate ($Na_3C_6H_5O_2.2H_2O$):**
   - Trisodium citrate is the anticoagulant of choice in blood tests for disorders of coagulation.
   - **Mode of action:** Acts as a chelating agent (inactivates calcium ions)
   - **Preparation:** 3.8% solution is prepared in distilled water and then sterilized.

   Uses: Citrated blood, citrate and blood in the ratio of 1:9, is used for coagulation studies, and for ESR test by the Westergren's method in the ratio of 1:3. Along with other components, sodium citrate is used for storing donated blood in blood banks, since it can be safely given intravenously. Oxalates are toxic and cannot be given intravenously.
3. **Double oxalate mixture:**
   - This is a mixture of ammonium oxalate and potassium oxalate in the ratio of 3:2 is an effective anticoagulant. It is thus called double oxalate.
   - **Mode of action:** Oxalates prevent clotting by forming insoluble calcium salts, thus removing ionic calcium.
   - **Preparation:** A large number of containers can be prepared at a time by placing 0.2 mL of oxalate mixture (3.0 g of ammonium oxalate and 2.0 g of potassium oxalate in 100 mL of distilled water) in each container and drying in gentle heat in an oven. This amount is sufficient for 8–10 mL of blood. Too much oxalate is hypertonic and damages all blood cells, while too little will not prevent clotting.
   - **Uses:** Though each oxalate by itself (also sodium and lithium oxalate) can prevent clotting, a mixture is used. Sodium oxalate should not be used since it causes crenation of red cells. Ammonium salt should not be used in urea and non-protein nitrogen tests. It is used in the tests where the cell volume should remain unaffected, e.g., ESR, PCV.
4. **Sodium fluoride:**
   - A mixture of 10 mg of sodium fluoride and 1 mg thymol is an anticoagulant as well as a preservative when a blood sample has to be stored for a few days. Since fluoride inhibits glycolytic enzymes (thus preventing loss of glucose), it is employed when plasma glucose is to be estimated.
5. **Heparin:**
   - Heparin, a highly charged mixture of sulfated polysaccharides, and related to chondroitin, has a molecular weight ranging from 15,000–18,000 and is a naturally occurring powerful anticoagulant. Theoretically, heparin is an ideal anticoagulant since no foreign substance is introduced into the blood.
   - It is normally secreted by mast cells that are present in many tissues, especially immediately outside many of the capillaries in the body. Both mast cells and basophils release heparin directly into blood. Heparin is also a cofactor for the lipoprotein lipase—the clearing factor.
   - Commercial heparin is extracted from many different tissues and is available in almost pure form (it was first extracted from the liver—hence the name "heparin"). Low megawatt fragments (mw 5000) have been produced from unfractionated heparin and are being used clinically since they have a longer half-life and produce more predictable results.
   - **Mode of action and uses:** Heparin by itself has no anticoagulant activity. However, when it combines with **antithrombin III,** the ability of the latter to remove thrombin (as soon as it is formed) increases hundreds of times. The complex of these two substances removes many other activated clotting factors—such as IX, X, XI, and XII.
   - **Preparation:** The required amount of stock solution is taken in a number of containers and dried at low heat. At a concentration of 10–20 IU/mL blood, it does not change red cell size and their osmotic fragility. It is, however, inferior to EDTA for general use. It should not be used for leukocyte counts, as these cells tend to clump. It also imparts a blue tinge to the background of blood films.

     Uses: It is used for estimation of blood gases, pH assay, and osmotic fragility. Clinically, it is used to prevent intravascular clotting of blood.
6. **Acid-citrate-dextrose and citrate-phosphate dextrose-adenine:**
   - Acid-citrate-dextrose (ACD) and citrate-phosphate-dextrose-adenine (CPD-A) are the anticoagulants of choice for storing donated blood in blood banks.
   - The CPD-A mixture is preferred as it preserves 2–3 DPG better.

Anticoagulants for In Vivo Use

The two in vivo anticoagulants are heparin and coumarins. Patients at increased risk of forming blood clots in their blood vessels, e.g., leg veins during prolonged confinement to bed, or during long flights, are sometimes put on these drugs (e.g., warfarin) to prevent thromboembolism. Their BT, CT, and PT are checked from time to time to adjust the dosage of the drug.

1. **Heparin** is particularly used during open-heart surgery in which the blood has to be passed through a heart-lung

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machine; or the dialysis machine during hemodialysis in kidney failure, and then back into the patient.

2. **Dicoumarol and warfarin:** The coumarin derivatives are vitamin K antagonists and thus inhibit the action of this vitamin that is essential as a cofactor for the synthesis of six glutamic acid-containing proteins—namely, factors II (prothrombin), VII, IX, and X, protein C, and protein S. The action of this anticoagulant is, however, slower than that of heparin.

Important: Anticoagulant therapy should not be confused with thrombolytic agents employed for dissolving blood clots.

QUESTIONS

1. **What measures are taken to prevent infection during venipuncture and skin-pricking?**
2. **What are the sources and main differences between venous blood and capillary blood? Why is capillary blood called peripheral blood?**
3. **What is the difference between plasma and serum? How will you get a sample of each?**
4. **What precautions will you observe to prevent hemolysis of venous blood samples?**
5. **What are anticoagulants? What is meant by in vivo and in vitro anticoagulants?**
6. **Why are the thumb and little finger not pricked for blood?**
7. **What are the sites for collecting capillary blood in infants?**
8. **Why should the pricked finger not be squeezed? What is meant by free-flowing blood?**

1.3: HEMOCYTOMETRY

STUDENT OBJECTIVES

After completing this experiment, the student should be able to:

- Describe the principle underlying hemocytometry.
- Describe the counting chamber and the dimensions of different squares on the counting grid.
- Name the diluting fluids and their composition.
- Identify the red blood cell (RBC) and white blood cell (WBC) pipettes, name their parts, and the differences between them.
- Name the precautions to be observed during dilution of blood in a pipette.
- Calculate the dilution obtained with each pipette.
- Describe the possible sources of error during dilution of blood.
- Charge the Neubauer's chamber and name the precautions observed during charging.
- Focus the counting grid for RBC and WBC under low and high magnifications and identify and count the cells.
- Explain the possible sources of error for cell counting.

INTRODUCTION

Hemocytometry is the procedure of counting the number of cells in a sample of blood; the red cells, the white cells, and the platelets being counted separately. It is assumed that the cells are homogeneously mixed (suspended) in the plasma in all regions of the body. However, even under physiological conditions, there are slight differences (e.g., higher red cell counts in venous and capillary blood than in arterial blood) which, though minor, are accentuated by muscular exercise, changes in posture, meals, etc. Nevertheless, important clinical information can be obtained if cell counts are done carefully on a venous blood sample.

PRINCIPLE

- Since the number of blood cells is very high, it is difficult to count them even under the microscope. This difficulty is partly overcome by diluting the blood to a known degree with suitable diluting fluids and then counting them.
- The sample of blood is diluted in a special pipette and is then placed in a capillary space of known capacity (volume) between a specially ruled glass slide (counting chamber) and a coverslip. The cells spread out in a single layer which makes their counting easy.
- Knowing the dilution employed, the number of cells in undiluted blood can then easily be calculated.

UNITS FOR CELL COUNTING

The result of cell counting is usually expressed as "so many cells per cubic millimeter (cmm; mm^3; µL) of blood". For example, RBC count—5.0 million/mm^3; WBC count—5,000 cells/mm^3.

The SI unit, however, is cells per liter of blood.
$1\ mm^3 = 1\ \mu L = 10^{-6}\ L$, $1\ \mu L \times 10^{-6} = 1\ L$

APPARATUS

1. **The counting chamber (hemocytometer):** It is a thick glass slide, appropriately ruled with a counting grid, i.e., squares of varying dimensions.
2. **The diluting pipettes:** Two different glass capillary pipettes, each having a bulb, are provided for counting RBCs and WBCs (these pipettes are sometimes called *cell pipettes* or blood pipettes. The third pipette that the students will be using is the hemoglobin pipette, which does not have a bulb).

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3. **Coverslips:** Special coverslips having an optically plane and uniform surface should be preferred over ordinary coverslips.
4. Red blood cell (RBC) and white blood cell (WBC) **diluting fluids.**
5. Watch glasses, spirit swabs, blood lancet/needle, etc.

Counting Chamber: Improved Neubauer Chamber

- The counting chamber was introduced by Crammer in 1805. Its modification by Thoma, and later by Neubauer remained in use for a long time. Improved Neubauer chamber is in current use.
- The counting chamber **(Figs. 9A and B)** is a single, solid, and heavy glass slide. This is divided into three parallel platforms (pillars or flanges) separated from each other by two shallow trenches (moats, gutters, or troughs). Thus, there are two lateral platforms and one central platform.
- The central platform or "floorpiece" (sometimes also called the plateau) is wider, and exactly 0.1 mm (one-tenth of a mm) lower than the two lateral pillars. The floorpiece is divided into two equal parts by a short transverse trench in its middle as shown in **Figures 9A and B**. Thus, there is an H-shaped trench or trough enclosing the two floorpieces.
- Identically ruled areas, called "counting grids", consisting of squares of different sizes, are etched on each floorpiece. The two counting grids allow RBC and WBC counts to be made simultaneously if needed, or duplicate samples can be run.
- The two lateral platforms support a coverslip which, when in position, will span the trenches and provide a capillary space 0.1 mm deep between the undersurface of the coverslip and the upper surface of the floor pieces.

Note: Hemocytometers with silver-coated floorpieces show the grids beautifully, which makes them easier to use by the students.

Old Neubauer Chamber

There are nine 1 mm squares. The four corner groups of 16 squares each are for WBC counting, while the central 1 mm^2 area has 16 groups of 16 squares each for RBC counting (rather than 25 groups of 16 smallest squares as in improved Neubauer chamber). The medium squares are separated by triple lines.

The Counting Grid

The ruled area on each floorpiece, the counting grid, has the following dimensions:

- Each counting grid **(Fig. 10)** measures 9 mm^2 (3 mm × 3 mm). It is divided into nine large squares, each 1 mm^2 (1 mm × 1 mm).
- Of these nine squares, the four large corner squares are lightly etched, and each is divided by single lines into 16 medium-sized squares each of which has a side of 1/4 mm, and an area of 1/16 mm^2 (1/4 × 1/4 mm). These four large corner squares are employed for counting leukocytes and are, therefore, called WBC squares **(Fig. 10)**.

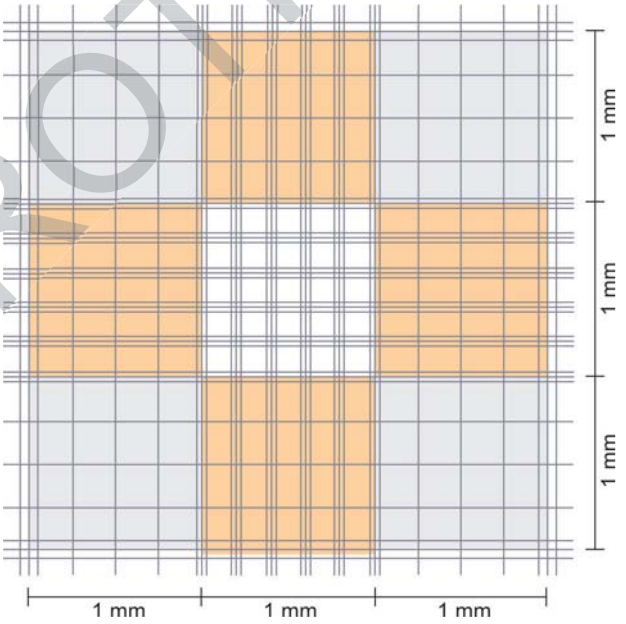
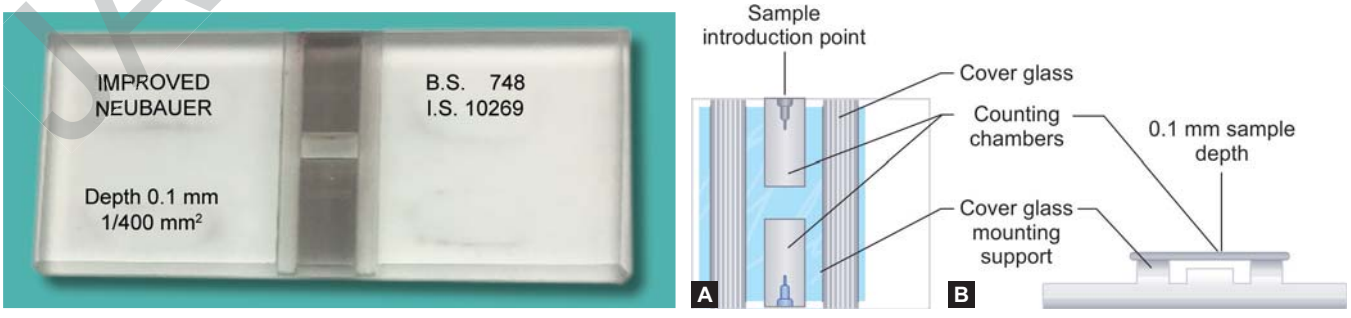


FIG. 10: The counting grid of improved Neubauer's ruling. The four corner groups of 16 squares (side of each square = 0.25 mm) are used for counting WBCs. The RBCs are counted in five groups of 16 smallest squares each (side of each smallest square = 0.05 mm).
(RBCs: red blood cells; WBCs: white blood cells)



FIGS. 9A AND B: Hemocytometer (Improved Neubauer's Chamber) (A) Surface view (B) Side view.

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- The central densely etched large square (1 mm × 1 mm), called the RBC square, is divided into 25 medium-sized squares, each of which has a side of 1/5 mm.
- Each of these medium squares is set off (separated) from its neighbors by very closely placed triple lines (tram lines). These triple lines extend in all directions beyond the boundaries of the 9 mm^2 ruling, i.e., in between all the WBC squares around the central RBC square.
- Each of the 25 medium squares (side = 1/5 mm), bounded by triple lines (which are 0.01 mm apart), is further divided into 16 smallest squares by single lines **(Fig. 17)**. Thus, each smallest square has a side of 1/5 × 1/4 = 1/20 mm (0.05 mm), and an area of 1/400 mm^2.

Study of the Diluting Pipettes

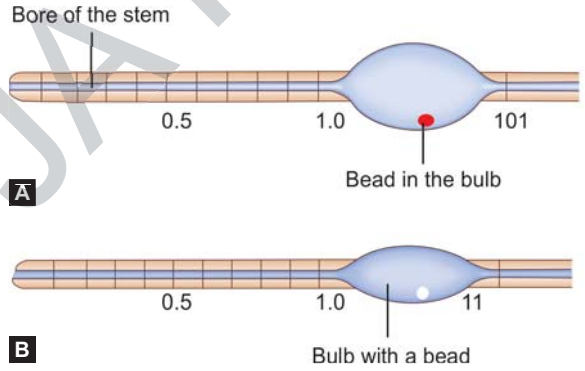
1. Red blood cell pipette
2. White blood cell pipette.

Figures 11A and B shows the two glass capillary pipettes used for diluting the blood. Each pipette has a long narrow stem (for measuring the blood), which widens into a bulb (for diluting the blood/which in turn, leads to a short stem).

Parts of a Diluting Pipette

- **The stem:** The long narrow stem has a capillary bore and a well-grounded conical tip. It is divided into 10 equal parts (graduations) but has only two numbers etched on it—0.5 in the middle of the stem, and 1.0 (or 1) at the junction of stem and the bulb.
- **The bulb:** The stem widens into a bulb which contains a free-rolling bead—red in the RBC pipette, and white in the WBC pipette. The bead helps in mixing the blood and the diluent and also helps in quick identification at a glance. It also tells whether the pipette is dry or not (it rolls freely if it is dry). The dilution of the blood occurs in the bulb only, and since the volume of the bulb is known, it is possible to dilute the blood with a diluent in accurately known proportions. The bead does not affect the dilution as the volume of the bead is taken into consideration during manufacturing of the pipette.
- **Rubber tube and mouthpiece:** The bulb narrows again into a short stem to which a long, narrow, and soft rubber tube bearing a mouthpiece (often red in RBC pipette, and white in WBC pipette) is attached. The rubber tube is 25–30 cm long to facilitate filling of the pipette by gentle suction. It also allows the pipette to be held horizontally so that one can comfortably watch the blood or diluting fluid entering the pipette.

Just beyond the bulb, the number 101 is etched on the RBC, and 11 on the WBC pipettes.



FIGS. 11A AND B: The diluting pipettes (blood pipettes). (A) RBC pipette: it has three markings—(1) 0.5, (2) 1.0, and (3) 101; (B) WBC pipette: it has three markings—(1) 0.5, (2) 1.0, and (3) 11.
(RBC: red blood cell; WBC: white blood cell)

Principle Underlying the Use of Diluting Pipettes

- It is important to understand that the numbers marked on the pipettes—0.5, 1.0, and 101 on the RBC pipette and 0.5, 1.0, and 11 on the WBC pipette—do not indicate absolute or definite amounts (or volumes) in terms of so many cubic millimeters (or μL), a mistake commonly made by the students.
- These figures only indicate relative volumes (parts)/or relative volumes in relation to each other. That is, half volume (from tip to mark 0.5), one volume (from the tip to mark 1.0) in both pipettes; and 11 volumes (from tip to mark 11 above the bulb in WBC pipette), and 101 volumes (from tip to mark 101 in RBC pipette). Of course, all these volumes will have certain definite volumes in terms of cubic millimeter, but we are not concerned with these but only the relative volumes or parts in relation to each other.
- It can be seen that the capillary bore in WBC pipette is wider than that in RBC pipette, and therefore, will hold more blood though the volume of the stem in both cases is 1.0 (one).

Differences between Red Blood Cell Pipette and White Blood Cell Pipette

The differences between RBC pipette and WBC pipette are described in **Table 4**.

Filling the Pipette (Fig. 12)

- Place a drop of anticoagulated blood on a glass slide or get a fingerprick under aseptic conditions.
- Holding the mouthpiece of the pipette between your lips and keeping the pipette (with its graduations facing you) at an angle of about 40° to the horizontal, place

Table 4: Differences between RBC pipette and WBC pipette.

RBC pipette	*WBC pipette*
Calibrations are 0.5 and 1.0 below the bulb, and 101 above the bulb	Calibrations are 0.5 and 1.0 below the bulb, and 11 above it
The capillary bore is narrow, thus it is a ***slow-speed pipette***	The capillary bore is wider, hence it is a ***fast-speed pipette***
Bulb is larger and has a red bead	Bulb is smaller and has a white bead
The volume of the bulb is 100 times the volume contained in stem	The volume of the bulb is 10 times the volume of the stem

(RBC: red blood cell; WBC: white blood cell)

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FIG. 12: Filling the pipette.

its tip within the edge of the drop. Gently suck on the mouthpiece and draw blood until it is just above the mark 0.5 (capillary action cannot fill the pipette at this angle). If more than the required amount of blood is drawn, the pipette is gently tapped to the palm or the tip of the pipette is touched against a nonabsorbent material in order to bring the blood to desired mark.

- The blood drop should be of adequate size (say 3–4 mm in diameter). If it is too small or if the tip is lifted out of the drop, air will enter the pipette along with blood. If the tip presses against the skin, the bore at the tip will get blocked and no blood will enter the stem even if you suck hard at the mouthpiece.
- Alternately, the pipette (after removing the rubber tube) may be filled with blood (without sucking/ by lowering its bulb end below the horizontal) and allowing the blood to flow down the stem by gravity.

- Remove the pipette from the blood drop and clean its outer surface with a cotton swab by wiping it toward the tip. Do not touch the bore at the tip otherwise some blood will be pulled out.
- Keeping the pipette horizontal all the time, bring the blood in the stem to the exact mark 0.5 by wiping the tip on your palm (or on a paper) a couple of times till the blood recedes to the exact mark.

Note: Do not use filter paper for this purpose as it will absorb a large amount of blood, and neither should you try to blow out the extra blood.

- Holding the pipette nearly vertical, immerse its tip in the diluting fluid taken in a watch glass, and suck the diluent to the mark 11 (WBC) or 101 (RBC). As the fluid is sucked up, the blood is swept before it into the bulb of 10 volumes (WBC) or 100 volumes (RBC pipette). The pipette is tapped with the tip of the index finger to knock the bead down below the surface of the solution in the bulb to prevent formation of bubbles.
  - The sucking up of diluting fluid should not be done very quickly because blood being viscous, if a sufficient time is not allowed, a thick film of blood will remain sticking to the inside of the capillary bore, thus introducing a significant error.
  - The dilution of blood should not be delayed otherwise it is likely to clot in the stem.
  - Once the diluent has been taken to the appropriate mark, keep the pipette horizontal so that the fluid does not run out by gravity.
  - Do not place the pipette on the table, or delay the mixing because it becomes impossible to dislodge the cells from the walls of the bulb once they settle down.
- Mixing the blood with the diluting fluid: Once the diluting fluid has been sucked up, press the tip of the pipette against the palm of the other hand and rotate it for 3–4 minutes so that the blood and diluent get thoroughly mixed.

  Alternately, remove the rubber tube, close the pipette ends with the thumb and forefinger of your right hand, and shake it vigorously with a figure of eight motion.

  Do not shake the pipette with an endwise motion as this will force the cells out of the bulb into the stem.
- **Charging the chamber:** Once the blood and the diluent have been mixed well, "charge" the chamber **(Fig. 13)**. After the blood has been diluted in the pipette, the stem contains only the cell-free diluent. This fluid has, therefore, to be discarded before the chamber can be filled.
- Charging the chamber requires patience, practice, and understanding of how to correctly judge the size of the drop, the angle at which the pipette should be held on the floorpiece, and the time needed for filling (charging) the chamber. This is called the "speed of the pipette".

  Obviously, it varies with the size of the capillary bore in the stem of the pipette.
  - **High-speed pipette:** Since the bore of the WBC pipette is wider, a drop will form more quickly at its tip, and it will be larger, as compared to the RBC pipette. This requires that this pipette should be held more horizontally, say, at an angle of 10–20° and for a shorter time.
  - **Slow-speed pipette:** The bore of the RBC pipette being narrow, it will take a longer time for a suitable



FIG. 13: Charging a chamber.

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drop to form. It should, therefore, be held at a steeper angle—say, 60–70°.

It is for this reason that the students should first practice charging a chamber with the RBC pipette and then with the WBC pipette.

Calculation of Dilution Obtained (Dilution Factor)

When blood is sucked up to the mark 0.5 (half part or volume) and is followed by the diluting fluid, the blood enters the bulb first and is followed by the diluent to the mark 101 (RBC pipette), or mark 11 (WBC pipette). The stem in both pipettes contains only the diluent. Thus, *the dilution of the blood occurs in the bulb only.*

Red blood cell pipette:

- Since the volume of the bulb is 100 (101 – 1.0 = 100), it means that 100 volumes (or parts) of diluted blood contain 0.5 (half) part of blood and 99.5 (100 – 0.5 = 99.5) parts or volumes of diluents.
- This gives a dilution of 0.5 in 100 (half in hundred), or 1 in 200 (one in 200), the dilution factor being 200 (the blood will be diluted 200 times). Similarly, if blood is taken to the mark 1.0 followed by diluted to mark 101, the dilution now would be 1 in 100.

White blood cell pipette:

- In this case, the volume of the bulb is 10 (11 – 1 = 10). When blood is taken to the mark 0.5 (half part or volume) followed by diluent to the mark 11, the volume of the diluted blood is now 10, which contains 0.5 part of blood and 9.5 parts or volumes of the diluting fluid.
- This gives a dilution of 0.5 in 10 (half in ten), or 1 in 20 (one in 20), the dilution factor being 20 (the blood will be diluted 20 times). Similarly, if blood is taken to the mark 1.0 followed by diluted to mark 11, the dilution now would be 1 in 10.

For Red Blood Cell Counting

The red cells are counted in four corner groups and one central group of medium squares, each of which has 16 smallest squares, i.e., in a total of 80 smallest squares.

Area of smallest square = 1/20 mm × 1/20 mm = 1/400 mm^2.

Since the depth of the chamber is 1/10 mm, the volume of the smallest square = 1/400 × 1/10 = 1/4,000 mm^3.

Note: The RBC pipette can be used for counting platelets, WBCs (when their number is very high, as in some leukemias), or spermatozoa in the semen.

For White Blood Cell (Total Leukocyte) Counting

This count is done in the four corner groups of large squares, each of which has 16 medium squares.

Area of one medium = 1/4 mm × 1/4 mm square = 1/16 mm^2. Volume of this square = 1/16 mm^2 × 1/10 mm = 1/160 mm^3.

Note: The WBC pipette can be used for diluting the blood for counting RBCs in cases of severe anemia, or for counting platelets. It can also be used for counting sperms and bacteria.

Focusing the Counting Grid

Examine the grid on each floorpiece, without the coverslip, under low and high magnifications. Rack the condenser up and down, closing/adjusting the diaphragm at the same time. When properly focused, the rulings (lines) appear as translucent darkish lines. Since the grid lines, the cells lying on the floorpiece, and those sticking to the underside of the coverslip are located at different levels in the 0.1 mm space, all of them may not be seen clearly at one focusing position. It is for this reason that 'racking' of fine adjustment is required.

- With low magnification of 100 times, one large square, 1 × 1 mm is visible in one field, i.e., a group of 16 medium squares (for WBC counting), or a group of 25 medium squares (for RBC counting).
- Examine the squares under high magnification.

PROCEDURE

1. Assuming that the blood and the diluent have been properly mixed, the next step is to charge the chamber. Place a coverslip on the chamber so that it spans the floor pieces and the trenches around them—a process called "centering" the coverslip.
2. Roll the pipette once more between your palms to mix the contents of the bulb.
3. Keeping your finger over the top of the pipette and releasing it in a controlled manner, allow the first two drops to drain by gravity. This fluid contains cell-free diluting fluid in the stem which has not taken any part in the dilution of blood.
4. Hold the pipette at an appropriate angle (depending on the speed of the pipette) and watching carefully, allowing a drop of diluted blood to form at its tip. Then quickly place the tip of the pipette on the floorpiece in gentle contact with the edge of the coverslip. As the surface of the drop touches the coverslip, the fluid will run under it by capillarity and form a uniform film. Lift the pipette as soon as the floorpiece is covered with diluted blood.
5. The chamber should be charged at one go and not in parts. For this, one needs a drop of proper size. Both sides of the chamber need to be charged so as to reduce the error in counting. Charging on one side may lift the coverslip unevenly on that side. Charging the two sides keeps the cover slip uniformly placed. If the cover slip is not uniformly placed, the distance between the chamber and the cover slip will be higher than 0.1 mm, and the cell counts will not be accurate.
6. Try to achieve an ideally-charged chamber. Before placing the chamber on the microscope stage, wait for 2–3 minutes to allow the cells to settle down.

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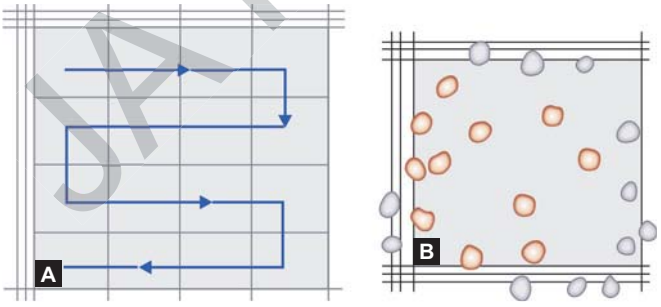
Important: *Ideally-charged chamber*: An ideally-charged chamber is completely filled with diluted blood. If any blood flows into the trenches, it is called "overcharging". If the fluid is insufficient to cover the floorpiece, or if there are air bubbles, it is called "undercharging" (air bubbles are formed if the coverslip or the floorpiece is dirty with grease or is moist).

- *Effects of overcharging:* When the chamber is overcharged the fluid will overflow into the gutters. This may give false low results as the cells will enter the gutter and settle there.
- *Effects of undercharging:* In an undercharged chamber fluid does not cover the entire central platform. There may be no cells in the peripheral squares thus giving the false low results.
- If there is over or undercharging, wash the chamber and coverslip in soap and water, dry them, and recharge the chamber.

7. Once the chamber has been properly charged, move it to the microscope. Wait for 2–3 minutes so that the cells settle down. Counting cannot be started when the cells are moving and changing places due to currents in the fluid. *Counting the cells*: Focus the appropriate squares under the required magnification and start counting as described in **Figure 14**.

Rules of counting **(Figs. 14 A and B)**:

1. Care should be taken not to count the same cells again. For this one has to follow the rules of counting.
2. Counting is done in the direction as indicated by the arrow in **Figure 14A**.
3. Count the cells present in the square and on its left and lower lines are counted and those on its right and upper lines are excluded. This is **'L pattern'** counting as shown in **Figure 14B**.
4. However, **"inverted L pattern"** can also be used i.e., count the cells lying within a square and those lying on or touching its upper horizontal and left vertical line and those cells on its right and lower lines are ignored as they will be counted in the adjacent squares **(Fig. 18B)**. Arrows in the figure indicate the squares to which the cells belong.
5. Whichever pattern is chosen, it is best to follow it for the entire counting.



FIGS. 14A AND B: Counting of cells. (A) Direction of counting as indicated by arrow; (B) Cells to be counted as shown in the smallest square bounded by triple lines. It is to be noted that In 'L pattern' counting the black colored cells are counted and white colored cells are not counted.

6. In squares surrounded by triple lines, the *middle line* is considered the boundary of that square **(Fig. 14B)**, and the same rules of counting apply. It is important to recapitulate here that the smallest RBC square is either surrounded by a single line all around or it may be bound by triple lines on its one or two sides only while a medium sized RBC square is surrounded by triple lines all around **(Fig. 17)**.
7. While counting the cells, continuously "rack", the fine adjustment up and down so that cells sticking to the underside of the coverslip are not missed.

Sources of error in cell counting:

1. Pipette error
2. Dilution error
3. Chamber
4. Statistical
5. Field error.

Note: Three important sources of error: (1) pipette error, (2) chamber error, and (3) field error can produce a variation of as much as 10–15%, or even more in the hands of a student. On the other hand, hemoglobin and packed cell volume are easy to determine and give enough information about the blood picture.

PRECAUTIONS

1. The pipette should be clean and dry and the bead should roll freely.
2. The pipette should not be lifted out of the blood drop while filling it with blood, otherwise air will enter it.
3. The drawing up of diluent, after blood has been taken in the stem, should not be delayed, otherwise it will clot in it. If the blood has clotted, the pipette is kept in strong nitric acid for 24 hours. A flexible suitably thick metal wire is used to clean the capillary bore after washing the pipette in running water. The process may have to be repeated.
4. The pipette should be cleaned soon after the experiment is over.
5. Do not touch blood other than your own.
6. Ensure that the counting chamber and the coverslip are absolutely clean, grease-free, and dry.
7. While charging the chamber ensures that it is neither under nor overcharged.
8. Never bring the objective lens down while looking into the microscope as the chamber is likely to be scratched or broken.

Note: Automatic Electronic Cell Counters
The electronic cell counter uses a volumetric impedance method. An electrolyte solution (diluent) containing suspended blood cells is aspirated through the aperture. Two electrodes are located close to the aperture and constant current flows between them. When a blood cell passes through the aperture, the resistance between the electrodes momentarily increases and a very small voltage change occur corresponding to the resistance. The voltage signal is amplified and sent to the electronic circuit. The data is then corrected by the CPU and displayed on the screen.

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QUESTIONS

1. What is the principle underlying the use of a counting chamber?
2. What are the dimensions of WBC and RBC squares?
3. What are the features of a properly charged chamber? How will over- or undercharging of the chamber affect the red cell count?
4. What is the function of the coverslip?
5. How does the improved Neubauer chamber differ from the older variety of Neubauer chamber?
6. Why is it difficult to see grid lines and cells clearly at the same focus?
7. How will you identify a red cell pipette and a white cell pipette?
8. What is the function of the bead in the bulb?
9. What are the units of markings on the pipettes?
10. What is the function of the bulb in a diluting pipette?
11. Why is it important to discard the first two drops of diluted blood from the pipette before charging the counting chamber?
12. How will you clean a pipette when blood has clotted in the stem?
13. Can a pipette be used for any other purpose than cell counting?
14. How is the entry of air bubbles prevented while diluting the blood?
15. If the blood is sucked above the 0.5 mark of the pipette how is it brought down?

1.4: THE RED CELL COUNT

STUDENT OBJECTIVES

After completing this experiment, the student should be able to:

- Describe the relevance of doing red cell count.
- Identify the red blood cell (RBC) pipette; fill it with blood and diluent.
- Charge the counting chamber and count the red cells.
- Describe the composition of diluting fluid and the function of each component.
- Give the normal RBC count in different age groups.
- Describe the site and stages of erythropoiesis, and factors that regulate it.
- Explain the causes of anemia and polycythemia.

INTRODUCTION

PY2.11: Estimate Hb, RBC, TLC, DLC, blood groups, BT/CT, RBC indices.

Red blood cells are the most abundant cells in the peripheral blood. The human RBC is normally a circular, non-nucleated, biconcave disc containing hemoglobin. The biconcave shape aids in exchange of oxygen and carbon dioxide maximally and also helps it to withstand osmotic lysis and to easily pass through narrow capillaries (diapedesis).

Red cell dimensions:

- **Shape:** Biconcave disc
- **Size:** 7.4 (7–8) micron in diameter
- **Thickness:** 2.0 micron
- **Surface area:** 140 square micron.

The formation of red blood cells is known as erythropoiesis.

Sites of formation:

- Fetal life—spleen, liver, thymus, and bone marrow
- Soon after birth—red bone marrow
- Adult life—long bone cavities and femur.

STAGES OF ERYTHROPOIESIS

The whole process of maturation requires 3–5 days and is mediated by interleukins and GM-CSF that influence the secretion of erythropoietin **(Flowchart 1)**. Average lifespan of RBCs is 120 days.

Functions

- To help in exchange in oxygen and carbon dioxide between lungs and tissues.
- Breakdown products of RBC, e.g., globin maintain the protein storage pool of the body.
- Storage of iron is needed for hemoglobin synthesis.

Normal Values

Adults:

- **Males:** 5.2 (4.5–6) million per mm^3 of blood.
- **Females:** 4.7 (4.0–5.0) million per mm^3 of blood.
- **Newborns:** 6–8 million per mm^3 of blood.

Methods of counting:

- Manual
- Automated

Manual Method

Principle

The blood is diluted 200 times in a red cell pipette and the cells are counted in the counting chamber.

Knowing the dilution employed, their number in undiluted blood can easily be calculated.

Apparatus

1. **RBC pipette:** It should be clean and dry and the bead should roll freely **(Figs. 15 and 16)**.

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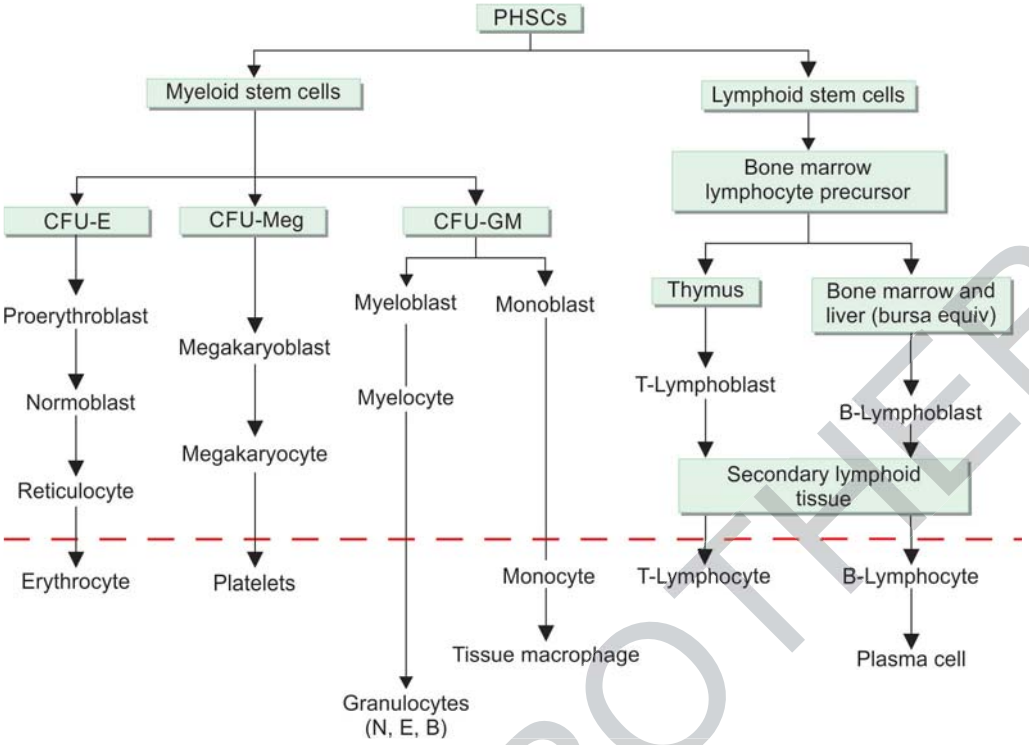
FLOWCHART 1: Hematopoiesis—development of blood cells. Cells above dotted line: Development and maturation in bone marrow. Cells below dotted line: Cells present in blood.



(CFUs: Colony forming units are committed (progenitor) cells; PHSCs: Pluripotent hematopoietic stem cells)

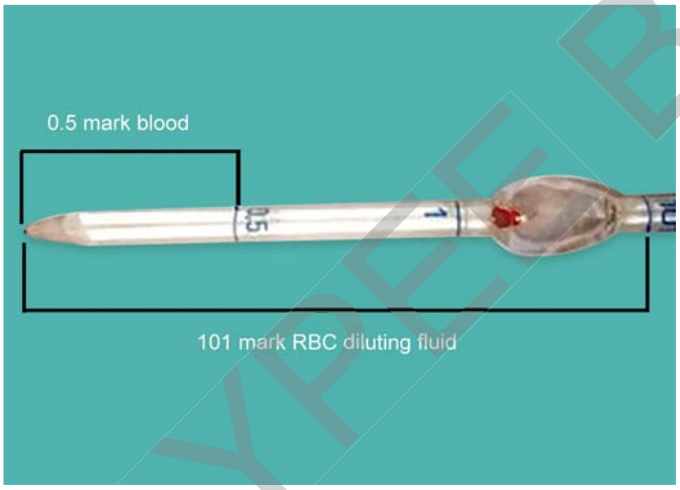


FIG. 15: RBC pipette.

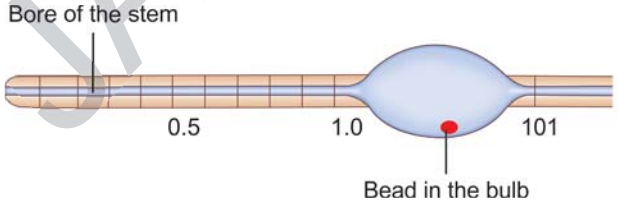


FIG. 16: The RBC pipette. It has 3 markings—0.5, 1.0, and 101.

2. **Improved Neubauer chamber with coverslip:** These should be clean and dust free.
3. Microscope with LP and HP objectives and 10× eyepiece.
4. Disposable blood lancet/pricking needle:
   - Sterile cotton/gauze swabs.
   - 70% alcohol/methylated spirit.

Hayem's fluid (RBC diluting fluid): The ideal fluid for diluting the blood should be isotonic and neither cause hemolysis nor crenation of red cells. It should have a fixative to preserve the shape of RBCs and also prevent their autolysis.

Composition of Hayem's fluid: Sodium chloride (NaCl): 0.50 g, Sodium sulfate (Na_2SO_4): 2.50 g, Mercuric chloride ($Hg Cl_2$): 0.25 g, Distilled water: 100 mL.

- Sodium chloride and sodium sulfate provide isotonicity so that the red cells remain suspended in diluted blood without changing their shape and size.
- Sodium sulfate also acts as an anticoagulant, and as a fixative to preserve their shape and to prevent rouleaux formation (piling together of red cells).
- Mercuric chloride acts as an antifungal and anti-microbial agent, prevents contamination and growth of microorganisms.

Note:

- **Dacie's solution:** This diluting fluid is an alternative to Hayem's solution. It is simple to prepare and keep for a long time. It contains 3.13 g of trisodium citrate, 1.0 mL of 37% formalin (commercial formaldehyde), and distilled water to 100 mL.
- **Normal saline:** 0.9% sodium chloride solution can be used if Hayem's or Dacie's fluids are not available. However, the red cells have to be counted within an hour or so of filling the pipette. Also, the RBCs are likely to form rouleaux. Further, a stock solution of normal saline cannot be kept for this purpose.

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Procedure

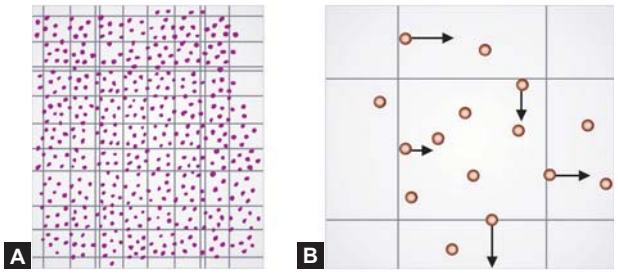
- Place about 2 mL of Hayem's fluid in a watch glass.
- Examine the chamber with the coverslip "centered" on it, under low magnification. Adjust the illumination and focus the central 1 mm square (RBC square on the counting grid) containing 25 groups of 16 smallest squares each **(Fig. 17)**. All these squares will be visible in one field. Do not change the focus or the field. Admitting too much light is a common cause of the inability to see the grid lines and squares clearly.
- Move the chamber to your worktable for charging it with diluted blood (it can be charged while on the stage, but it is more convenient to charge it on the table).
- **Filling the pipette with blood and diluting it:** Get a fingerprick. Wipe the first two drops of blood and fill the pipette from a fresh drop of blood up to the mark 0.5. Suck Hayem's fluid to the mark 101 and mix the contents of the bulb for 3–4 minutes as described earlier.
- **Charging the chamber:** Observing all the precautions, fill the chamber with diluted blood.
- Since the RBC pipette is a slow-speed pipette, it will need to be kept at an angle of 70–80° while charging the chamber.
- Move the chamber to the microscope and focus the grid once again to see the central 1 mm square with the red cells distributed all over.
- Wait for 3–4 minutes for the cells to settle down because they cannot be counted when they are moving and changing their positions due to currents in the fluid. During this time draw a diagram showing the RBC square. Then draw five groups of 16 square each, showing their relative positions—the four corner groups and one central group for entering your counts.
- **Counting the cells:** Switch over to high magnification (HP lens) and check the distribution of cells. If they are unevenly distributed, i.e., bunched at some places and scanty at others, the chamber has to be washed, dried, and recharged.
- Move the chamber carefully and bring the left upper corner block of 16 smallest squares in the field of view (there are no smallest squares above and to its left).
- An occasional leukocyte may be seen. It is larger than a red cell, appears refractile (shiny) and granular, it also contains a nucleus. Since the ratio of WBC to RBC is 1:600–700. Therefore, hardly any WBCs are counted. Since in healthy individuals, the total number of RBCs counted in 80 smallest squares (5 × 16) is approximately 600, WBCs do not affect the RBC count. But if it is counted with the red cells, it will increase the count by 10,000/mm^3 since the multiplication factor is 10,000 for cells in 80 smallest squares.
- Dust particles may be present on the objective, eyepiece, or in the diluent. They are irregular in size and shape. If they are on the eyepiece, they will rotate with the eyepiece. Red cells, on the other hand, are round discs, of uniform size, light pink in color which is lighter in the center.

Rules for Counting (Figs. 18A and B)

- See chapter on hemocytometry for details.
- An occasional WBC (may be 1 in 600–700 RBCs) may be seen appearing grayish and granular but it is not to be counted with the red cells.
- The counting is to be done in 80 smallest squares, i.e., in 5 blocks of 16 squares each.

Observations and Results

RBC in the RBC square under high power (Fig. 19): Add up the number of cells in each of the 5 blocks of 16 smallest squares. A difference of more than 20 between any 2 blocks indicates uneven distribution.



FIGS. 18A AND B: (A) Microscopic view of a charged chamber showing even distribution of red cells. A group of 16 smallest squares is shown in the middle; (B) 'Inverted L pattern' for cell counting: Count the cells lying within a square and those lying on or touching its upper horizontal and left vertical line cells lying or touching its lower horizontal and right vertical lines are to be omitted as they will be counted in the adjacent squares. Arrows indicate the squares to which the red cells belong.

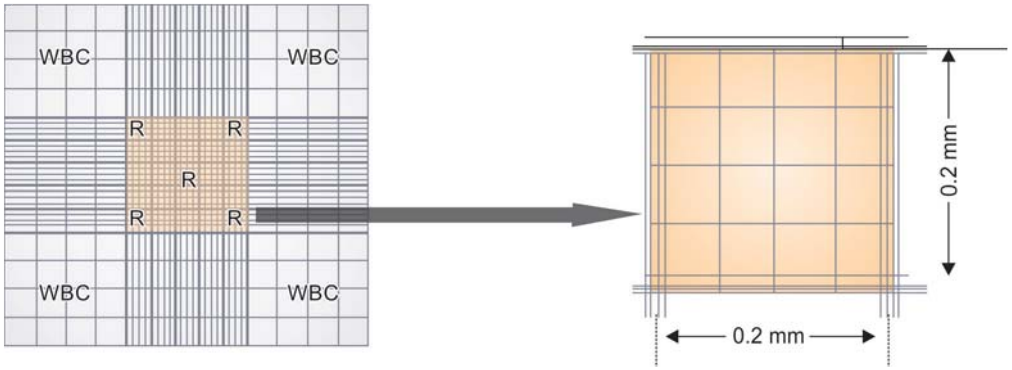


FIG. 17: Neubauer's chamber showing RBC squares under low power (10X). On the right is RBC square as seen under high power (40X).

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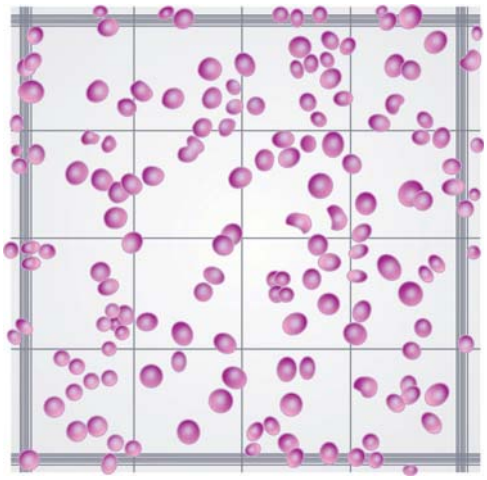

FIG. 19: Red blood cells in the RBC square.

- **Calculation of dilution obtained (dilution factor).** Refer **Experiment 1.3:** Recall that the dilution with this pipette is **1 in 200** as blood is taken to mark 0.5.

  Thus, the dilution factor is $= \frac{\text{Final volume attained (100 parts)}}{\text{Volume of blood taken (0.5 part)}}$
- **Calculation of volume of fluid examined:** We know the count in 80 smallest squares which have a volume (space) of 1/50 mm^3. We can also know the cell count in 1 smallest square which has a volume (space) of 1/4,000 mm^3.
- **Calculation of red cell count**
  Let N be the number of cells in 1/50 mm^3 of diluted blood.
  Cells in 1 mm^3 of diluted blood = N × 50 dilution employed was = 1 in 200
  Therefore, number of cells in 1 mm^3 of undiluted blood will be = N × 50 × 200 = N × 10,000.
  Thus, adding, 4 zeros in front of N will give the RBC count per 1 mm^3 of undiluted blood.

Example

Number of cells in 80 smallest squares = 480
These cells are present in 1/50 mm^3 of diluted blood. Dilution employed is = 1 in 200
Number of cells in 1 mm^3 of undiluted blood will be:
= 480 × 50 × 200
= 480 × 10,000
= 480,0000, i.e., 4.8 million/mm^3.

Sources of Error

As mentioned in **Experiment 1.3,** despite all precautions in the procedures the degree of error with this method is said to be about ±15%. Thus, with a count of 5.0 million/mm^3, the technical error of a single count comes to about ±0.75 million/mm^3. And in the hands of a student, this error can be as high as 20%. The following factors are responsible for the error:

- **Pipette error:** It may be due to inaccuracy of graduations, the method of filling with blood and diluent, or mixing.
- **Chamber error:** The counting grid and the depth of the chamber may not be accurate, or the process of charging it may introduce an error.
- **Field error:** The distribution of cells on the grid may not be uniform.

Notes:
- ❑ To be of any value, the test must be carried out as meticulously as possible.
- ❑ Repeated counts of a charged chamber do not give significantly better results.
- ❑ Taking repeated samples of blood, counting the cells in each, and taking their mean value can eliminate pipette error. But this has hardly any practical value.

Normal Red Cell Count

- Express your result as million/mm^3
- The average cell counts and their ranges are:
  - *Males* = 5.0 million/mm^3 (4.75–6.0 million/mm^3)
  - *Females* = 4.5 million/mm^3 (4.0–5.5 million/mm^3).

Precautions

1. Observe all precautions described for getting a finger prick, filling the pipette with blood and diluent, and charging the chamber.
2. Clean the chamber with a lint-free piece of cloth. Once cleaned, do not touch the central part. Any oils left from your fingers on the coverslip or the chamber are fatal to cell counting.
3. If there is over or undercharging, wash, clean, dry, and refill the chamber.
4. Continuously rack the fine adjustment while counting the cells.
5. Do not draw diluents directly from the stock bottle as it may contaminate the solution with cells.
6. Discard the sample and recharge the chamber if the distribution is not uniform.
7. Follow the rules of counting to avoid the counting twice.

Physioclinical Significance

There are number of physiological and pathological conditions where RBC count can be altered.

1. **Decrease RBC count**
   - Physiological causes:
     - Pregnancy
     - RBC count is lower in children than adults
     - Females have lower RBC count than males because of estrogen that depresses erythropoiesis by decreasing erythropoietin.
   - Pathological: Anemia
     Hemodilution, e.g., excess ADH secretion as in posterior pituitary tumors.
2. **Increase in RBC count**
   - Physiological causes:
     - High altitude
     - Newborns
     - Hemoconcentration, e.g., excessive sweating.
   - Pathological:
     - Hemoconcentration, e.g., diarrhea, vomiting
     - Chronic hypoxia, e.g., congenital heart disease, chronic obstructive pulmonary disease
     - Polycythemia vera.

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QUESTIONS

1. **Why is blood diluted 200 times for red cell count?**
2. **Will the bead in the bulb affect the dilution?**
3. **How will you clean the chamber after your experiment is over?**
4. **If Hayem's solution is not available, can you use any other solution?**
5. **Why should both sides of the chamber be charged?**
6. **How will you differentiate red cells from dust particles?**
7. **Why is it necessary to follow the rules of counting?**
8. **What are the sources of error in this experiment?**
9. **What is the fate of leukocytes in this experiment?**
10. **What is the site of formation of red cells during fetal life and after birth?**

Sites of erythropoiesis:

- **Fetal life—**spleen, liver, thymus and bone marrow.
  - In the early weeks of embryonic life, primitive, nucleated red blood cells are produced in the yolk sac (mesoblastic phase).
  - During the middle trimester of gestation, the liver is the main organ for production of red blood cells, but reasonable numbers are also produced in the spleen and lymph nodes (hepatic phase).
  - During the last month or so of gestation and after birth, red blood cells are produced exclusively in the bone marrow (myeloid phase).
- **After birth till 5 years of age—**red bone marrow
- **Adult life—**long bone cavities and femur.

11. **What is the lifespan and fate of red cells?**

The average lifespan of red cells as determined by radioactive and agglutination methods is about 120 days (about 40 days in macrocytic anemia and spherocytosis). During their lifetime, they circuit the cardiovascular system some 300,000 times (their elastic framework, formed by the protein spectrin, permits them to regain their biconcave shape as they emerge through the capillaries). As they squeeze through 5–6 µm capillaries again, they are subjected to severe mechanical stress, wear and tear of their plasma membranes. Since there is no nucleus and other organelles, the red cells cannot synthesize new components to replace the damaged ones. The plasma membranes become more and more fragile with age; they are now more likely to burst particularly as they squeeze through the narrow trabecular spaces of the spleen.

About 10% of the worn-out RBCs fragment in the circulating blood. These fragments and old cells are removed from the blood and destroyed by the fixed phagocytic macrophages in the spleen, liver, etc. (the spleen has been called the graveyard of RBCs). The breakdown products, globin and iron of heme are recycled for reuse by the body.

12. **What is erythropoiesis? What are the stages of differentiation and maturation of red cells?**
13. **How is erythropoiesis regulated?**

The proliferation and differentiation of various cells in the bone marrow is very accurately controlled by following factors:

Various hormones or factors (hemopoietic growth factors) that include: colony stimulating factors (CSFs), cytokines, interleukins (ILS), erythropoietin (EPO), etc. **Factors that regulate erythropoiesis:**

1. ***Erythropoietin (EPO):*** The critical balance between RBC production and destruction is maintained by adjustments in the circulating levels of EPO. Its blood level is greatly increased in anemia. The number of erythropoietin-sensitive stem cells in the bone marrow is greatly increased by EPO. These are then converted into RBC precursors and then to mature erythrocytes.
   Erythropoietin is a glycoprotein that contains 165 amino acid residues and 4 oligosaccharide chains. About 95% of EPO is secreted by the peritubular interstitial cells of the kidneys, and 15% from the perivenous hepatocytes of liver. Hypoxia is the most potent stimulator for EPO secretion. Cobalt salts and androgens also stimulate EPO secretion.
2. ***Grade I proteins:*** Proteins of animal origin, soya beans, etc.
3. ***Vitamins:*** B_{12}, folic acid, pyridoxine, other B complex vitamins, and vitamin C are needed for red cell formation.
4. ***Trace metals:*** Iron, copper, zinc, and cobalt are required in trace amounts.
5. ***Hormones:*** Androgens, thyroxin, growth hormone, and cortisol are required.

14. **Which physiological conditions cause a decrease in RBC count?**
15. **What is anemia and what are its causes?**

See chapter on Estimation of Hemoglobin.

16. **What is polycythemia and what are its causes?**

Polycythemia: It refers to an increase in the number of red cells above the normal level. Erythrocytosis is a better term to describe an absolute increase in the total red cell mass.

1. **Polycythemia vera (erythremia, or primary polycythemia):** A gene abnormality in the early red cells causes them to form more and more cells. Erythropoietin production is not raised, but may be decreased.
2. **Secondary polycythemia:** Hypoxia due to lung diseases, congenital heart disease, and cardiac failure increases the RBC count.
3. **Polycythemia due to hemoconcentration:** Fluid loss during severe vomiting and diarrhea and loss of plasma in burns cause polycythemia. There is no increase in total red cell mass.

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Physiological polycythemia is seen during residence at high altitudes, and in newborns. Emotional stress and severe exercise may cause a temporary increase in RBC count.

17. What is meant by "stem cell harvesting" and what is their practical value?

Transplantation of stem cells (confined mainly to red bone marrow) from normal persons to patients of certain leukemias and abnormal bone marrow, have been in use for many years. The abnormal bone marrow is first destroyed by drugs and whole body radiation, and bone marrow aspirated from the hip bone of a donor is then transfused into the patient. The normal stem cells (taken from the donor) then settle in the recipient bone marrow where they start to produce normal cells in due course of time.

The harvesting (collecting) of pluripotent stem cells (PHSCs) from the umbilical cord blood (where they are present in much larger numbers than in adult bone marrow) is now being employed. The PHSCs taken from the blastocysts of human embryos have been cultured and work on these cells may prove beneficial to currently incurable diseases like Parkinson's, Alzheimer's, diabetes mellitus, and repair of damaged heart muscle in coronary artery disease.

OBJECTIVE STRUCTURED PRACTICAL EXAMINATION-I

Aim: To dilute the given sample of blood 200 times using a pipette and diluent.
Procedural steps: See text above.
Checklist:

1. Selects the correct pipette and checks that it is clean, dry and patent. (Y/N)
2. Takes enough diluents in a watch glass and sucks blood to the exact 0.5 mark and sees that there are no air bubbles. (Y/N)
3. Wipes off blood sticking to the outside of the tip of the pipette. (Y/N)
4. Sucks diluting fluid exactly to the mark 101. (Y/N)
5. Holds the pipette horizontally between the palms and rolls it gently to mix the contents of the bulb. (Y/N)

OBJECTIVE STRUCTURED PRACTICAL EXAMINATION-II

Aim: To charge the Neubauer chamber with diluted blood provided to you in a RBC pipette.
Procedural steps: See text above.
Checklist:

1. Ensures that the chamber and coverslip are clean and dry. (Y/N)
2. Mixes the contents of the bulb between the palms. (Y/N)
3. Places the coverslip on the central plateau of the chamber to cover both the ruled areas. (Y/N)
4. Discards the first few drops from the pipette and allows a suitable-sized drop to form. (Y/N)
5. Places the tip of pipette on the chamber, touching the edge of the coverslip and allows the fluid to spread evenly over the counting grid without over- or under charging. Charges the other side also. (Y/N)

1.5: THE TOTAL LEUKOCYTE COUNT

STUDENT OBJECTIVES

After completing this experiment, the student should be able to:

- Indicate the importance of doing total leukocyte count (TLC) in a clinical setting and in practical physiology.
- Do the total leukocyte count by the manual method, and compare its degree of error with the error of RBC counting.
- Name the constituents of Turk's fluid and their functions.
- Indicate the precautions you will observe.
- Describe the normal TLC in different age groups.
- Name the different leukocytes, their site of formation, functions, and regulation of leukopoiesis.
- Define leukocytosis, leukopenia, and physiological and pathological conditions in which they are seen.

INTRODUCTION

PY2.11: Estimate Hb, RBC, TLC, DLC, blood groups, BT/CT, RBC indices.

The white blood corpuscles (WBCs, leukocytes) constitute the major defense system of the body against invasion by bacteria, viruses, fungi, toxins, and other foreign invaders. Their number is kept remarkably constant in health, but it increases or decreases in many diseases particularly acute and chronic infections. A clinician generally wants this test done along with differential count, hemoglobin, etc., as part of "complete blood count" (CBC) in cases of fever, especially if the cause of fever is not immediately apparent (pyrexia of unknown origin or PUO).

Normal White Blood Cell Count

Normal count**:** 4,000–11,000 cells/mm^3 of blood in adults. The count after birth may be as high as 18,000–20,000/mm^3, the normal levels being reached in a few years. In the adults, about 55–75% of the WBCs are granulocytes, while in young children, lymphocytes dominate. The count may be high in some physiological conditions such as heavy exercise, stress, etc.

STAGES OF LEUKOPOIESIS: SEE FLOWCHART 2

Leukopoiesis is the process of producing white blood cells (WBCs), also known as leukocytes that are formed and

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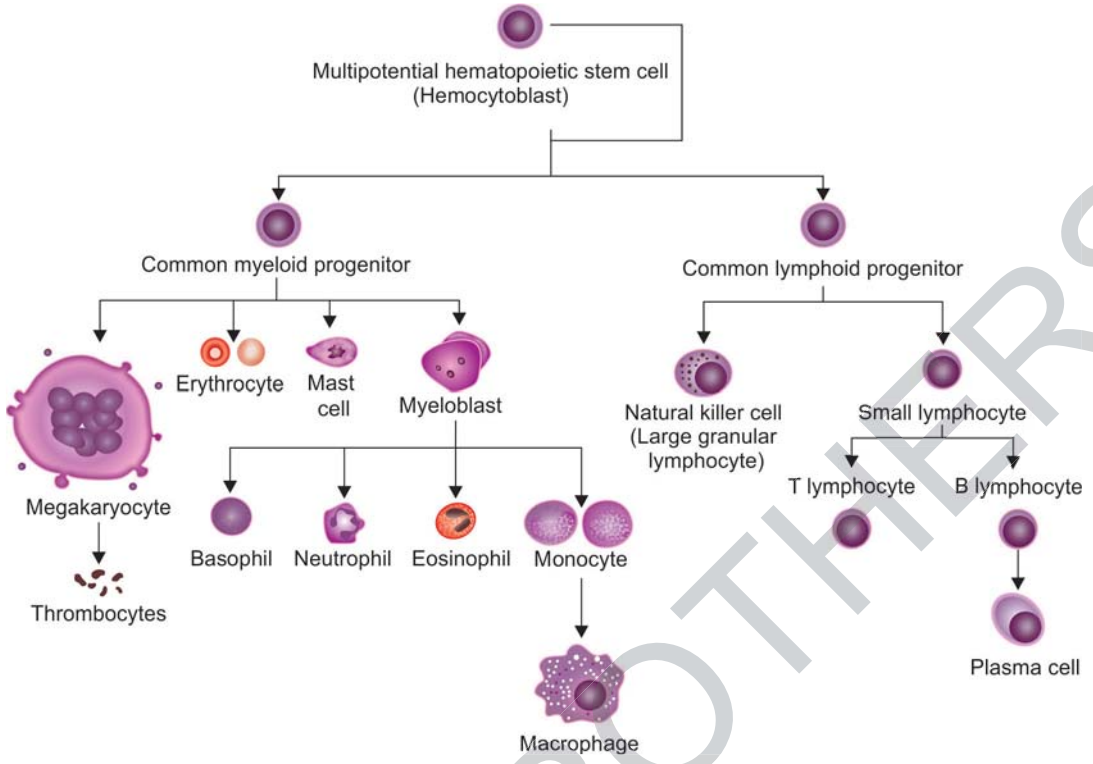
FLOWCHART 2: Stages of leukopoiesis.



matured in bone marrow located in bones in adults and hematopoietic organs in the fetus. White blood cells (WBCs) have a lifespan ranging from a few hours to several days, depending on the type, with granulocytes having a shorter lifespan (6–13 days) and lymphocytes potentially living up to 200 days.

TYPES OF LEUKOCYTES

The WBCs, unlike red cells contain nuclei but no hemoglobin. Depending on the presence or absence of clearly visible and conspicuous, chemical-filled granules (vesicles) in their cytoplasm (that are made visible by staining), they are grouped into two types: ***granular*** and ***agranular.***

- **Granulocytes:** There are three types of granulocytes that can be recognized under the compound microscope according to the coloration of their cytoplasmic granules—***neutrophils, eosinophils*** (eosin loving), ***and basophils*** (basic loving).
- **Agranulocytes:** In contrast to granulocytes whose nuclei are lobed, the nuclei of agranulocytes are not lobed but appear as a single mass. Although the cytoplasm contains chemical-filled granules, these are not visible under the light microscope due to their small size and poor staining with the usual dyes. The agranulocytes include—***monocytes*** and ***lymphocytes.***

CHIEF FUNCTIONS OF LEUKOCYTES

- The chief function of leukocytes is to provide immunity (protection) against various invaders and thus constitute an important mechanism of survival by preserving health and fending off disease. The immune system consists of task-specific cells that are in a constant state of vigilance and readiness—like the branches of armed forces. They recognize the invaders as "foreign" to the body and engage them in combat at the site of invasion.
- The **tissue macrophages** (that develop from blood monocytes and act as the "sentinels"), and neutrophils (they act as the "infantry" and are transported by blood to the site of invasion), respond most quickly and destroy the invaders by phagocytosing them (both these cells move through the tissues by active amoeboid movements and are attracted to the inflamed area). Thus, these two types of WBCs along with antimicrobial proteins (interferons—alpha, beta, and gamma, and complement system and natural killer lymphocytes, form the ***second line of defense)***. ***The first line of defense*** against infection are the surface barriers, i.e., intact skin and mucous membranes, that prevent the entry of pathogens into the body.

PRINCIPLE

A sample of blood is diluted with a diluting fluid which destroys the red cells and stains the nuclei of the leukocytes. The cells are then counted in a counting chamber and their number in undiluted blood reported as leukocytes/mm^3.

The volume of the fluid in the bulb is 10 times the volume of fluid in the stem, which can give a dilution of 1 in 10 or 1 in 20. In the RBC pipette, the volume of the bulb is 100 times the volume of the stem, which can give a dilution of 1 in 100 or 1 in 200. Since the count of leukocytes is in thousands/mm^3, the blood requires much less dilution as compared to red cell count which is in millions/mm^3.

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They have also published *Practical Physiology—A Student's Workbook* in 2019, which is being used as a practical manual for medical undergraduates all over India.

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