10.5005/jp-journals-10015-1495

REVIEW ARTICLE

Troubleshooters in Light Microscopy

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ABSTRACT

The aim of this study is to review the significance of parts and operating principle of compound light microscopy with emphasis on their troubleshooters. Light microscope has been an important diagnostic tool in scientific research over the years. The invention of the microscope by Anthony Leeuwenhoek has given rise to an interesting dimension in life sciences where great discoveries have been made to study various microorganisms and the structure of cells. Handling the microscope requires sound knowledge about its parts and their construction for effective functioning. There are innumerable faults regardless of the elegance of the microscope equipment or the experience level and skill of the user. Errors must be addressed carefully to detect the cause that may be due to poor specimen preparation or technical issues. The majority of photomicrography errors traced on the optical configuration of the microscope include improper illumination, use of the wrong filters, incorrect setting of the substage components and preservation, processing and mounting of tissue specimens, or microscopic optics. By capturing various photomicrographic images at different erronic modes of microscopic handling and artifactual appearances, the acceptable approach for the use of compound microscope has been emphasized with possible remedies to overcome them. Comprehension of the compound light microscopic components with causes and remedies of troubleshooters may lead to efficient handling of the instrument for investigative and diagnostic purposes.

Clinical significance: Good knowledge of the right usage of microscope parts is pivotal in routine laboratory investigations for the diagnosis and prognosis of pathologies and scientific research.

Keywords: Light, Microscopy, Troubleshooters.

How to cite this article: Lavanya A, Sowmya SV, Rao RS, Augustine D, Haragannavar VC, Nambiar S. Troubleshooters in Light Microscopy. World J Dent 2017;8(6):511-518.

Source of support: Nil

Conflict of interest: None

INTRODUCTION

The present lifestyle and environmental changes have paved way to new diseases, and their diagnosis poses a huge challenge. Although currently available diagnostic and prognostic techniques are sophisticated and advanced, pathologists have to rely on light microscopes even today. The microscope is derived from a Greek word wherein "mikro" means "small" and "skope" means "to look." This optical instrument consists of a lens for achieving a clear and magnified image of an object, i.e., not visible to the naked eye. The use of microscopes was initiated by the Romans who employed glasses instead of lenses. Then, with a discovery that an image could be obtained when sun rays were focused on special glasses, called magnifiers or burning glasses, made a difference in the field of pathology. In 1609, Galileo Galilei developed a compound microscope with a convex and concave lens.[1]

Scientists have a variety of optical instruments to perform routine laboratory procedures and research, but the compound microscope is the "workhorse" and commonly found in laboratories. The microscopes introduced so far produce a magnified image of the specimen and render the details visible to the human eye or camera. It is important to know the relation between the eyes and microscope. For an image to be viewed, it must be presented to the eye in colors (visible spectrum) and with varying degrees of light intensity. Although we maintain a good relationship between the eyes and microscope, there are other different sources that can hamper good image formation.[2]

A sound knowledge of the construction and limitations of the microscope is important for its efficient usage. An ill-adjusted, badly illuminated microscope can give completely misleading information about a particular disease.[1,3] There are different sources of error in setting up a microscope, and it is not easy to track their precise cause. Hence, for better diagnosis, it is important to know the construction and functions of each part to eliminate their limitations. This review focuses on the importance of appropriate usage of microscope parts and their troubleshooters.

PRINCIPLE OF MICROSCOPE

A compound microscope is an optical instrument consisting of two convex lenses which are used for magnifying very small objects. The two series of lenses are the objective lens and the eyepiece lens. The lens placed toward the object is represented as an objective lens, and the one closer to our eyes is eyepiece lens. The focal length of the objective lens is shorter than the eyepiece lens as it helps in receiving more light rays from an object and forms a

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bright image. Many compound microscopes are binocular and have two ocular lenses. A binocular type will have a prism, either in the head or in the body tube that helps to split the image and direct it to the eyepiece.[1,4]

WORKING PRINCIPLE

When light from the light source is passed through a thin transparent object, condensation of rays is brought about by the substage condenser through the numerical aperture. There is a substage condenser located between the stage and light source that helps in condensing the light rays. This type of light condensing and gathering capacity of the condenser is called the numerical aperture of the condenser. The first lens, called objective lens, collects the light passing through the object from the light source and then focuses it on forming a real image of an object inside the microscope. Then, the formed image is magnified by a second lens called as eyepiece lens and is perceived as "virtual image" by the observer. As the light passes directly from the source to the eye, wherein the field of vision is brightly illuminated, it is also called bright-field microscope (Fig. 1).[1,4,5]

FUNCTIONAL SIGNIFICANCE OF PARTS OF COMPOUND MICROSCOPE

Compound microscope is divided into three basic structural components. They are body, base, and arm. The body contains the optical parts, and base helps in supporting the microscope and contains the illuminator, whereas arm acts as the connection between body and head. It has also been categorized mainly into optical and mechanical parts based on their function.

- Optical parts include light source, diaphragm, filter, condenser, objective, and eyepiece (Fig. 2C).
- Mechanical parts include base, milled knobs, mechanical stage, rack stop, curved arm, nose piece, beam splitter (Fig. 3C), draw and body tubes.

When all these microscopic parts function effectively, the image obtained will be of superior quality (Fig. 4A).

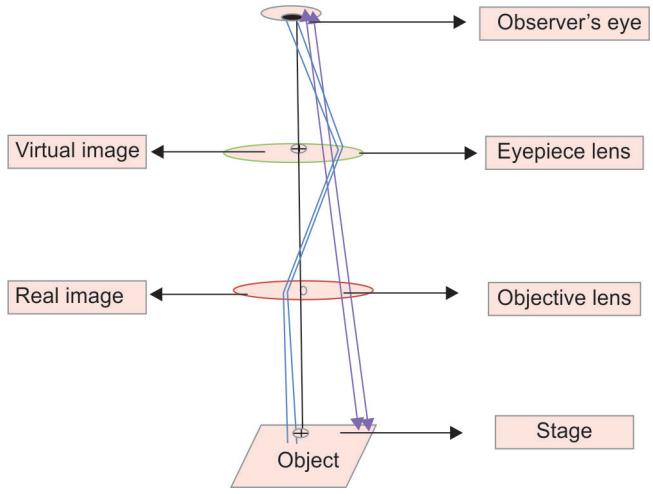


Fig. 1: Schematic diagram showing principle of compound light microscope

OPTICAL PARTS

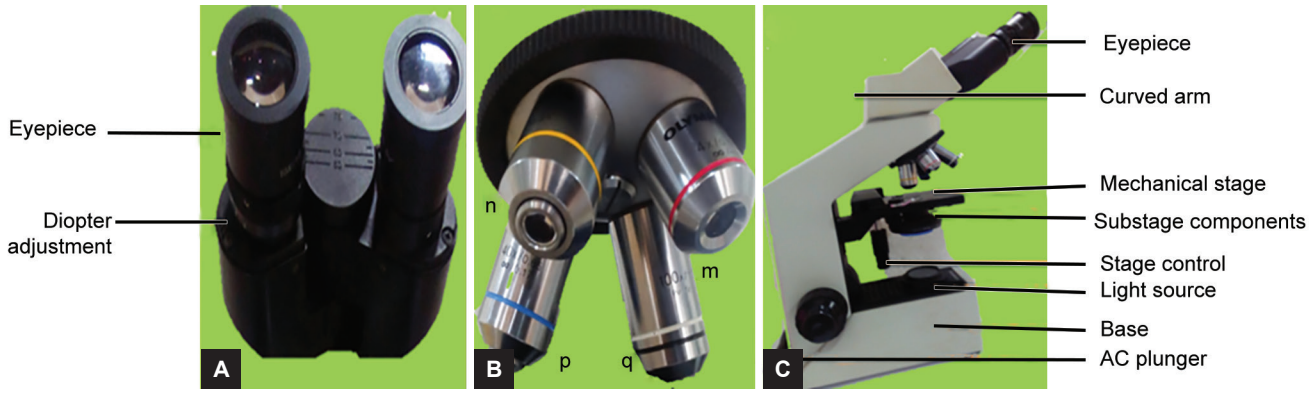
Light Source

The source is connected to the mains through a regulator that controls the brightness of the field. Choosing the appropriate light source for investigation in optical microscopy highly depends on the illumination strategy, specimen parameters, microscope configuration, and detector sensitivity.[1,4]

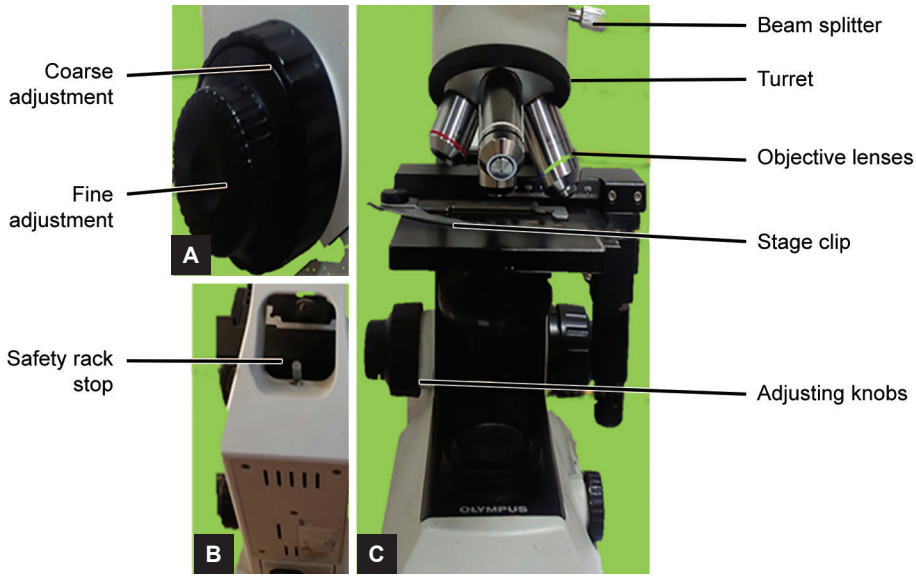
The causes for improper image formation due to the inadequate light source are a faulty connection of alternating current (AC) power cord, inoperative outlet, burned-out lamp, and blown fuse (Fig. 4B). This can be solved by the use of appropriate outlet connection, having qualified service and repair with the replacement of lamp and fuse.[6,7]

Diaphragm

The diaphragm is placed below the stage. It is used to control the amount of light reaching the specimen and angle of cone entering the objective.[1] The depth of the field, numerical aperture, and image quality are affected by the opening or closure of the aperture diaphragm.[1,4]



Figs 2A to C: Parts of the compound light microscope: (A) Eyepiece with diopter adjustment; (B) objectives with color coding, red 4× (m), yellow 10× (n), blue 40× (p), white 100× (q); and (C) optical parts of microscope



Figs 3A to C: (A) Photograph showing adjusting knobs; (B) safety rack stop; and (C) mechanical parts

The main aim is to achieve a balance between the resolution and contrast. If the aperture diaphragm is opened wide, the result will be a washed-out image with no contrast and difficulty in obscuring the details of the image. If there is over closure of aperture diaphragm, grainy image with less resolution will be obtained (Fig. 4C).[6-9]

Filter

Filters are placed in the path of light and help in increasing the contrast, blocking the ambient light, decreasing the intensity, and absorbing excess heat. A lamp with tungsten filament has a tendency to change their color and temperature depending on the light intensities. A filter placed over the lamp absorbs the red part of the spectrum and gives more neutral color.

There are various types of filters, such as neutral density, colored, color correcting, heat absorbing, and exciter filter.[1]

Neutral filters are used in decreasing the brilliance of illumination, i.e., most commonly used in photomicrography. If neutral density filter is not inserted into the light pathway, it results in increased intensity of light. This leads to color temperature imbalance between the light source and the film emulsion that produces an unexpected color shift.[1,4] If the color temperature of the light source is too low, orange-yellow-colored image is observed (Fig. 4D). This can be corrected by inserting the neutral density filters which provide uniform intensity and balanced color temperature over the entire microscopic field view.[6,7]

Condenser Lens

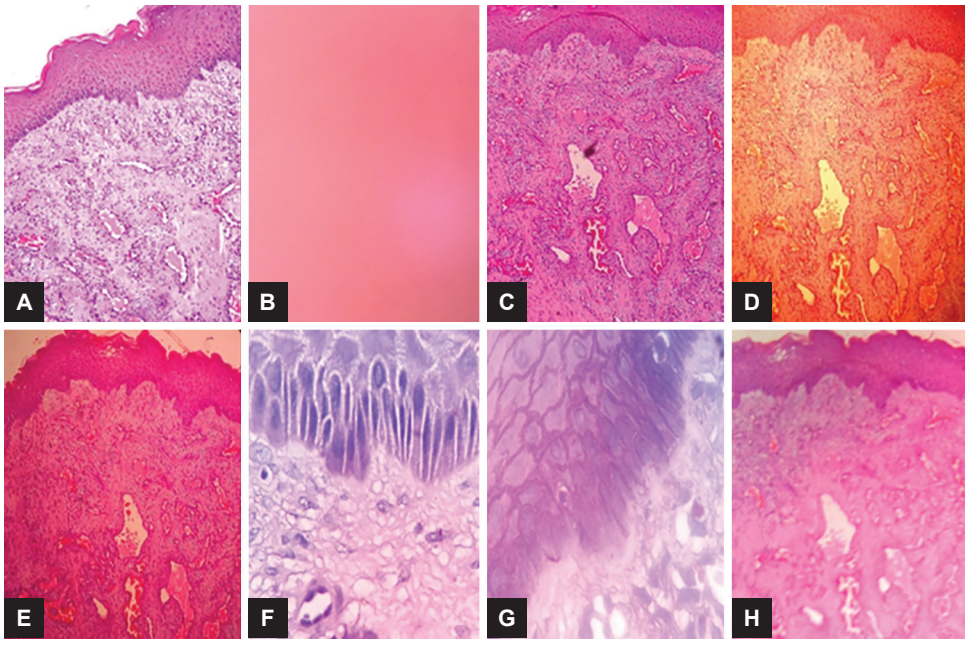
The condenser may be movable or fixed. When moved, it shifts in horizontal and vertical direction. It is a substage component that collects light from the light source and focuses it on the specimen.[1] Abbe, aplanatic, and achromatic are the three types of condensers. It provides sharper and clearer image and can be raised or lowered by turning the milled knobs/adjusting knobs. Most widely used is the Abbe condenser that produces a perfect image when a condenser with a lens system equal to that of the objective is selected.[1,5,6]

Unevenly illuminated objective field with poor image quality is formed when the condenser is being inappropriately lowered or moved away from the stage (Fig. 4E). Clear image with good intensity may be obtained by realigning the condenser and matching with the objective being used.[6,7,9]

Objective Lens

Objective lenses are those that are closer to the object. A compound microscope generally has four objective lenses with different magnifications and twined into a circular nose piece which may be rotated to select the desired magnification. The objective lenses are 4×, 10×, 40×, and 100× which are color-coded as red, yellow, blue, and white respectively, for rapid identification (Fig. 2B).[1,4,5] Table 1 summarizes the available objective lenses with their respective color codes.

Objective lenses are mainly of two types—achromatic and apochromatic that are responsible for magnifying the image of a specimen. The different colors of light while passing through the objective lens result in an image having colored fringes around them due to differences in wavelength. This effect can be prevented using achromatic and apochromatic objectives.[1,4]



Figs 4A to H: Photomicrographs of hematoxylin and eosin-stained tissues showing light microscopic operating errors (×100): (A) Optimal architectural and cellular details with ideal microscope construction; (B) light background without image; (C) grainy image by overclosure of the diaphragm; (D) orange-yellow-colored image; (E) unevenly illuminated image; (F) enhanced cellular features with oil immersion (×1,000); (G) unsharp image without oil immersion (×1,000); and (H) spotty image (×100).

Achromatic objectives are used for correcting the lens error of chromatic aberrations at two wavelengths—red and blue. Apochromatic objectives are made up of several fluorite lenses with glass lens and used for correction of chromatic aberration at three wavelengths—red, blue, and green.[1,4]

Dark spots and poor resolution in the focused image are due to the dirt or contamination with dust particles of the objective lens. This can be corrected by cleaning the lenses with lens paper.[6,7,9]

Oil Immersion Objective

Oil immersion is designed specifically to have the same refractive index as glass, and as a result, there is no bending of light between the objective and specimen. Oil immersion is used for viewing the bacteria, blood cells, and striations in skeletal muscle (Fig. 4F).

Hazy, unsharp, and poor resolution images are due to the use of 100× objective without oil, contamination of bottles containing cedarwood oil, and wrong immersion media (Fig. 4G). This can be prevented by placing the correct immersion medium without contamination. Clogging of the objective lens with oil can be cleaned using lens paper, and use of xylene should be avoided as it damages the lens.[6,7]

Eyepiece

An eyepiece consists of two convex lenses: One facing the objective called "field lens" and other placed near the observer's eye called "eye lens." These two lenses together are known as "ocular lenses" which have a power of magnification of about 10 times that of an objective and helps in magnifying the real image formed by the object (Fig. 2A). The eyepiece is the easiest component to rotate and get grotty in normal use.[1,10]

There are various types of eyepiece lenses which are

- Huygens—negative undercorrected and used with achromatic objectives
- Ramsden—positive and used for micrometry
- Widefield—provides large flat view
- High eyepoint—used by microscopists who wear spectacles
- Compensatory—used with apochromatic objectives

The veiled and spotty images produced may be due to dirt or oil on the eyepiece which can be identified by twirling the eyepiece (Fig. 4H). This can be corrected by cleaning the eyepiece with 70% isopropyl alcohol with lens paper.[6,7]

Diopter Setting

For optimal visualization, there are three main steps to be followed:

1. First, adjust the interpupillary distance and also binocular vision until the right and left fields of view concise completely. Record the value for future adjustment.[1]
2. Second, a control knob called diopter which is present only in the left eyepiece is adjusted. By looking

Table 1: Objective lenses with their color coding

Objective lens	*Color code*
5×	Red
10×	Yellow
16×	Green
20×	Green
25×	Turquoise
32×	Turquoise
40×	Light blue
50×	Light blue
60×	Cobalt blue
63×	Cobalt blue
100×	White
150×	White
250×	White
Oil immersion	Color code
Cedarwood oil	Black
Glycerol	Orange
Water	White

through the right eyepiece, first adjust the adjusting knobs to bring the specimen into focus.

3. Third, look into the left eyepiece and adjust the diopter ring to bring a fine focus (Fig. 2A).[1]

Eye fatigue results when there is incorrect diopter adjustment. This can be solved by correctly adjusting the diopter focus. Two separate or blurred images appear due to the incorrect interpupillary distance between the eyepieces and are corrected by adjusting the eyepieces (Fig. 5).[6,7,9]

MECHANICAL PARTS

Base

Base of the microscope is usually made up of durable material as it supports the microscope to stand and provides stability.[1,4]

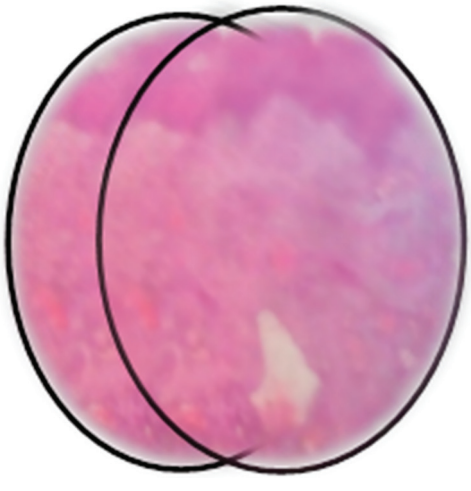


Fig. 5: Schematic diagram with two separate images of hematoxylin and eosin-stained tissue sections (×100)

Milled Knobs

There are two types of knobs—coarse- and fine-adjustment ones (Fig. 3A). Coarse adjustment is used to adjust the slide containing the specimen in back and forth direction to achieve a focused image. Fine adjustment helps to bring the specimen into the sharper focus.[1,4]

The unfocused and unsharp image occurs due to over tightening of the coarse adjusting knob as tension is extremely high (Fig. 6A). This can be prevented by loosening the ring properly for refocusing the image. If the focus obtained is lost immediately, it may be due to light tension. This can be corrected by rotating the knob clockwise to increase the tension to obtain the focused image.[6,7,9]

Mechanical Stage

A mechanical stage is a flat surface where the slide with the specimen is placed and moved in a side-to-side direction. It is fitted with Vernier scale with readings from 100 to 170 mm for recording the position of the slide in each direction. It is useful for repositioning of the slide at a later date for viewing a specific structure.[1,4]

Rack Stop

Safety rack stop is an adjustable screw situated between the stage and the arm (Fig. 3B), which prevents the stage from coming too far and ramming against the objective lens.[11,12] Improper vertical adjustment may lead to the formation of a light background or no image wherein the body of the microscope is either far away or too close to the object (Fig. 6B). This can be corrected by proper adjustment of the rack stop and by maintaining the required distance between the lens and the object.[9]

Curved Arm

The part of the microscope that holds the stage, body tube, and adjusting knobs and helps in carrying the microscope easily is the curved arm.[1]

Nosepiece/Turret

Nosepiece is the microscopic part that holds the objective lenses to view the specimen in different magnifications.[1] The partially illuminated objective field may occur due to the improper clicking of the revolving nosepiece (Fig. 6C). This can be solved by rotating the nosepiece till it gets clicked into its position.[6,7,9]

Beam Splitter

It is an optical device that splits a beam of light. Improper positioning of beam splitter results in partially illuminated object field which is due to horizontal malalignment (Fig. 6D).[9]

Draw Tube

It is the upper part of body tube slightly narrower and holds the eyepiece without getting slipped away during observation. It enables to adjust the mechanical tube length.[1]

Body Tube

The mechanical body tube length is the distance from the nosepiece opening where objective lenses are mounted till the top of observation tube where eyepieces are inserted. The accepted standardized body tube length is 160 mm (6.3 inches). When the objective and tube length are mismatched, ghost images are produced that are caused by converging light passing through the inclined plane glass surfaces. This can be corrected by maintaining optimal tube length.[1]

ACCESSORY COMPONENTS OF MICROSCOPE

Slide

The microscopic slide is a thin flat piece of glass that measures 75 × 26 × 1 mm thick. It is used to hold the specimen for microscopic examination.[1] Unfocused and steaminess of image are due to the placement of slide in upside-down position (Fig. 6E). This can be corrected by inverting the slide and viewing from the correct side.[9] Due to dust particles over the slide, dirty specks in the field of view can be observed which will interfere during examination and it moves when the slide is moved sideways (Fig. 6F). This can be corrected by cleaning the slide with soft sterile cloth.[9]

Coverslip

A coverslip or cover glass is a thin flat piece of transparent material which is square or rectangular in shape of about 0.17 mm thick and measures 25 × 75 mm. Coverslips are available with variable lengths and thickness. It is placed over the specimen in the slide which prevents drying out of specimen and protects from bacterial contamination.[1,4]

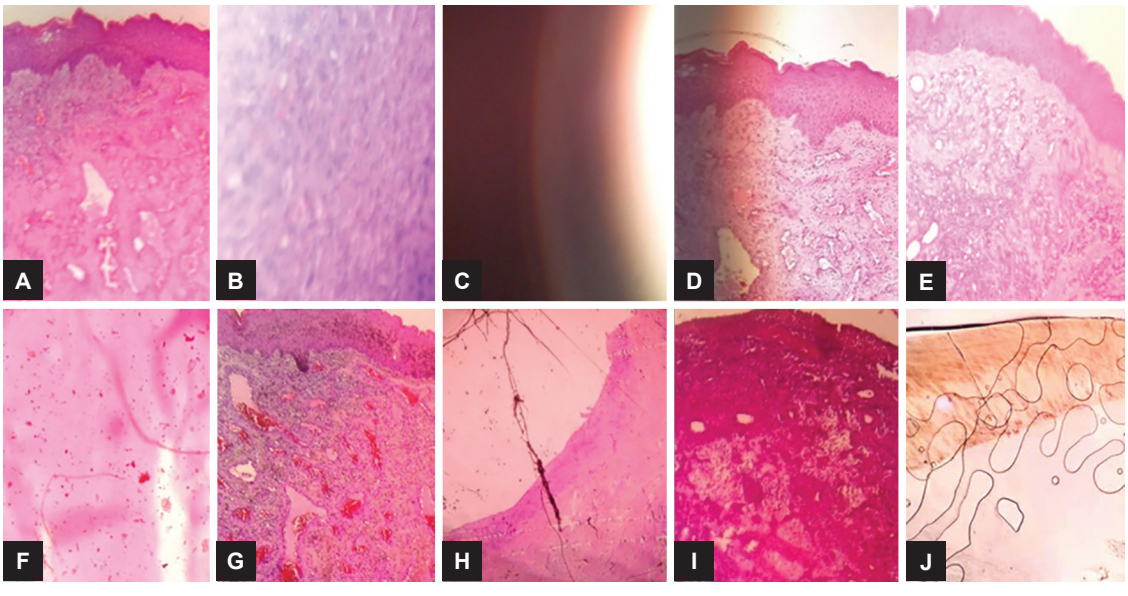
Cloudy image is formed due to the increased thickness of the coverslip that results in higher numerical aperture and loss of resolution (Fig. 6G). This can be corrected using type I coverslip for mounting. Misty and dark speck-like structures appear when mounting media are placed over the coverslip (Fig. 6H). This can be prevented by proper placement of mounting media and removing any excess medium that has flown over the slide or coverslip.[9]

Tissue Thickness

The specimen thickness ranges from 3 to 5 µm.[1] Due to the increased thickness of the specimen, the image formed appears distorted (Fig. 6I). This can be corrected using appropriate tissue thickness (4 µm).[9]

Mounting Media

Mounting media is the liquid, resinous, water-soluble solution in which specimen is embedded under the cover glass. Most commonly used mounting media are dibutyl pthalate polystyrene xylene, and its main function is



Figs 6A to J: Photomicrographs of hematoxylin and eosin-stained tissues showing light microscopic operating errors (×100): (A) Unsharp image; (B) hazy image due to improper safety rack stop; (C) partly illuminated image; (D) partially illuminated object field; (E) unfocused image due to improper slide placement; (F) dirty specks in field; (G) cloudy image; (H) speck-like structures caused by mounting media on coverslip; (I) distorted image; and (J) ground section showing air bubbles interfering visibility of tissues due to evaporation of xylene in mounting media

to protect the tissue. Ideally, the refractive index of the mounting media should be as close as to the tissue, which is approximately 1.53, that induces a certain amount of transparency and allows the stained tissue elements to be visible under microscope.[13]

When tissue section is placed without the mounting media, the image produced will be of poor clarity and contrast where the refractive index will be 1.0. This can be corrected by placing the tissue with proper mounting media.[13]

Air bubbles will appear when mounting media with evaporated xylene is used for the purpose of mounting. This can be prevented using a proper mounting media placed in an air-tight container where xylene evaporation is avoided (Fig. 6J).

SUMMARY

The various troubleshooters during the use of the light microscope, their causes, and remedies have been summarized in Table 2.

Table 2: Summary of troubleshooters, causes, and remedies in the usage of light microscope

Parts of microscope	*Problems*	*Causes*	*Remedies*
Eyepiece	Veiled and spotty image	Dirt or oil on the eyepiece, identified by twirling the eyepiece	Use 70% isopropyl alcohol and lens paper for cleaning the eyepiece
	Eye fatigue	Incorrect right and left diopter adjustment	Correct adjustment of the diopter focus
	Two separate or blurry images	Incorrect interpupillary distance between the eyepieces	Correct adjustment of the eyepieces
Adjusting knobs	Unfocused and unsharp image	Increased tension due to overtightening of the adjusting knobs	Loosen the ring properly for refocusing the image
	Focus obtained is lost	Decreased tension due to too much tightness of adjusting knobs	Rotating the knob clockwise to increase the tension to obtain focused image
Nosepiece (or) turret	Partially illuminated objective field	Revolving nosepiece has not been clicked to its position	Rotating the nosepiece till it gets clicked into its position
Safety rack stop	Dark background or no image	Improper vertical adjustment in which the body of the microscope is either far away or too close to the object	Proper adjustment of the rack stop helps in maintaining the required distance between the lens and the object
Beam splitter	An object field partially illuminated	Horizontal maladjustment created by improper positioning of beam splitter	Placement of the beam splitter in the proper position
Objective lens	Field of view cut off	Maladjustment of objective lens in which it is not clicked into the position	Positioning of objective lenses till it gets locked
	Dark spots and poor resolution in the focused image	Dirt or contamination particles in the objective lens	Cleaning the lenses using 70% isopropyl alcohol with lens paper
Oil immersion	Hazy, poor resolution with unsharp focused image	Without oil placed in 100× objective, contamination of bottles containing cedarwood oil and wrong immersion medium	Correct placement of immersion medium without contamination
Microscopic slide	Unfocused and steaminess of image	Slide placed in the upside-down manner	Inversion of the slide
	Dirty specks in the field of view	Dust particles over the slide	Cleaning the slide with soft sterile cloth
Coverslip	Cloudiness of image	Increased thickness of the coverslip more than 0.17 mm	Use type I coverslip for mounting
	Mistiness and dark specks like structures	Mounting medium is placed over the coverslip	Proper placement of mounting media
Tissue thickness	Distorted image	Increased thickness of specimen	Minimum thickness of 4 μm to be obtained
Condenser lens	Unevenly illuminated object field with poor image quality	Condenser lowered or moved away from the stage	Realigning the condenser
Diaphragm	Grainy image and less resolution	Overclosure of aperture diaphragm	Proper positioning of diaphragm
	Washed-out image and difficult to obscure the detail	Aperture diaphragm is opened wide	
Filter	Orange-yellow-colored image is obtained	If neutral density filter is not inserted into the light pathway, it results in increased intensity of light. This leads to color temperature imbalance between light source and film emulsion which produces unexpected color shift	Insertion of the neutral density filters which provide uniform intensity and balanced color temperature
Light source	No image	AC power cord not connected, outlet inoperative, lamp burned out, fuse blown	Connecting the outlet plug properly, have qualified service repair, replace lamp and fuse

CONCLUSION

A light microscope is a powerful tool that plays a central part in academics as an important teaching and learning aid, routine laboratory investigations for the diagnosis and prognosis of pathologies, and scientific research. It is described as an "extension of the senses" which aids our eyes to observe things that cannot be seen with the naked eye. The use of different light sources and combination of lenses make different types of microscopes suitable for different purposes. A sound knowledge of its components, physical parameters, and troubleshooters enables the user for its proper handling and precise use.

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