Ministry of Health of Ukraine
Ukrainian Medical Stomatological Academy

It is approved
on meeting of department of
medical informatics, medical and biological physics
27 August 2020
Minutes №1 of 27 August 2020

Head of department _____O.V.Silkova

Methodical instructions
for students' self-preparation work at preparation for a practical lesson
at home and at the classroom

Subject matter	**Medical and biological physics**
The unit	2. Bases of medical physics
Theme of lecture:	**Optical microscopy. Microscopy techniques.**
Year	1
Faculty	Medical
Speciality	Medicine

Poltava - 2020

The topic significance:

in biological researches, and in other branches of natural sciences, are connected to use of new physical methods. Discovery of optical microscope has given to such surprising turn of opening, thai its importance for a science are heavy for overestimating. In biology, at once after discovery of optical microscope in XVII century, was find out a structure of alive cell, the microorganisms, elements of blood etc. At the same time optical microscopy is one of main making of biophysical experiment.

Specific targets:

- To have general knowledge of the studied topic ;
- To understand, to remember and to use the received knowledge: optic laws, refraction in thin lenses, creation of images in lenses systems ;
- To master concepts of microscopic observation ;
- To seize habits of work with light microscope ;
- To seize technique of experiment on determination of microscopic object sizes ;
- To seize habits of work with object – micrometer and ocular micrometer ;
- To be able to carry out laboratory and experimental work .
- To measure sizes of microscopuc samples .

Basic knowledge, experience, skills necessary for studying the topic in connection with other subjects:

Disciplines	Obtainable skills
Previous (providing disciplines): physics, biology	To know basic concepts of optics: refraction in lenses, construction of image in lenses. To know special kinds of microscopy; electron microscopy, its kinds; atomic force microscopy. To describe them.

The subsequent disciplines: general biology; microbiology histology	Concept of microscopic studies. To use these concepts at the decision of tasks; Speak about this topic, to prepare sample to experiment.

Materials for the before-class self-preparation work:

List of main term, parameters, characteristics, which student have to learn at preparation to class:

Term	Definition
Resolution	**Resolution is the minimal distance on which there are two points shown to a microscope separately.**
Numerical aperture	The size n·sinφ, where n – refraction index of environment between a sample and an objective, φ – half of entrance angle of an objective (angle between extreme beams of the conic light beam which is entered in an objective). Than NA is more and the wavelength the less, the less resolved details of researched object.
Contrast of the image	Contrast of the image is a distinction of brightnesses of the image and a background.
Chromatic aberrations	Chromatic aberrations are caused by that light waves with different wave length are focused in different points on an optical axis. In result the image appears painted.
Spherical aberrations	Spherical aberrations are connected by that light which is taking place through the center of an objective, and light going through its peripheral part, is focused in different points on an axis. In result the image appears indistinct.
Achromatic objectives	In them chromatic aberrations are suppressed by means of glass elements with the different dispersion, extreme beams of a seen spectrum providing a convergence – dark blue and red – in one focus.
Apochromatic objectives	Objectives with the most complex color correction. In them not chromatic aberrations are only almost completely eliminated, but also correction of spherical aberrations is executed not for one, and for two colors.

Theoretical questions to class:

1. The key part of a light microscope.
2. Creation of image by objective and eyepiece
3. Rays pathes in microscope
4. Microscope magnification.
5. Resolving limit of a microscope.
6. Causes of aberrations appearance, methods of them reduction.
7. Polarization microscopy.
8. Luminescent microscopy.
9. Microphotographing and microfilming.
10. Dark-field microscopy.
11. Phase-contrast microscopy.
12. Microscopy with immersion.
13. Binocular microscopy
14. Electron microscopy.
15. Fiber optics, including use in medicine

Practice work executed at class:

Professional algorithms (instructions, reference cards) concerning mastering habits and

skills:

№	Maun tasks	Recommendations
1	2	3
1.	To seize a procedure of operation with the light microscope.	To familiarize with outward of the device, to determine the quantitative performances on an objective, an eyepiece; to examine an object-micrometer. To provide good illumination the subject stage.
2.	To determine the graduation mark (scale-division value) of an ocular micrometer.	An object - micrometer is glass or plastic plate with scale (squares). Scale-division value can be 1 mm or 0,01 mm usually. An eyepiece [ocular] micrometer is glass or plastic plate with linear scale. Ocular micrometer is allocated between ocular lenses in the plane of intermediate image formed by objective. In the ocular image of this scale is visible, and it overlaps sample image. To dispose an object-micrometer with the known value of division (M_{ob} =1 mm) on the stage. To determine, how many divisions (n) of an ocular micrometer are coated with particular number N of divisions of the image of a reference scale (object-micrometer): ocular micrometer scale-division value M_{oc} = N/n. Iterate 3 times. Calculate average value.
3.	To determine the size of a microscopic object.	To dispose a microscopic object on the stage, to receive its precise image, to sketch it, then with the help of the stage controls handles, to bring a crossroads, all over again on one, and then on the second end of a microscopic object. In both cases to make a scale reading of a micrometer m_1 and m_2; to $l = m_1 – m_2$. Calculate object size $L = l \times M_{oc}$. Execute measurings 5 times, bring results into the copybook in Table 1.

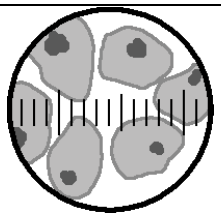

Fig.10. Ocular-micrometer scale against a background of cells.

Table 1

N	Object	N	n	M_{oc}, mm	l, mm	L, mm
1						
2						
3						
4						
5						

Contens of the topic.

Light microscope - the typical models use compound lenses and light to magnify objects images. The lenses refract the light, which makes the object beneath them appear closer.

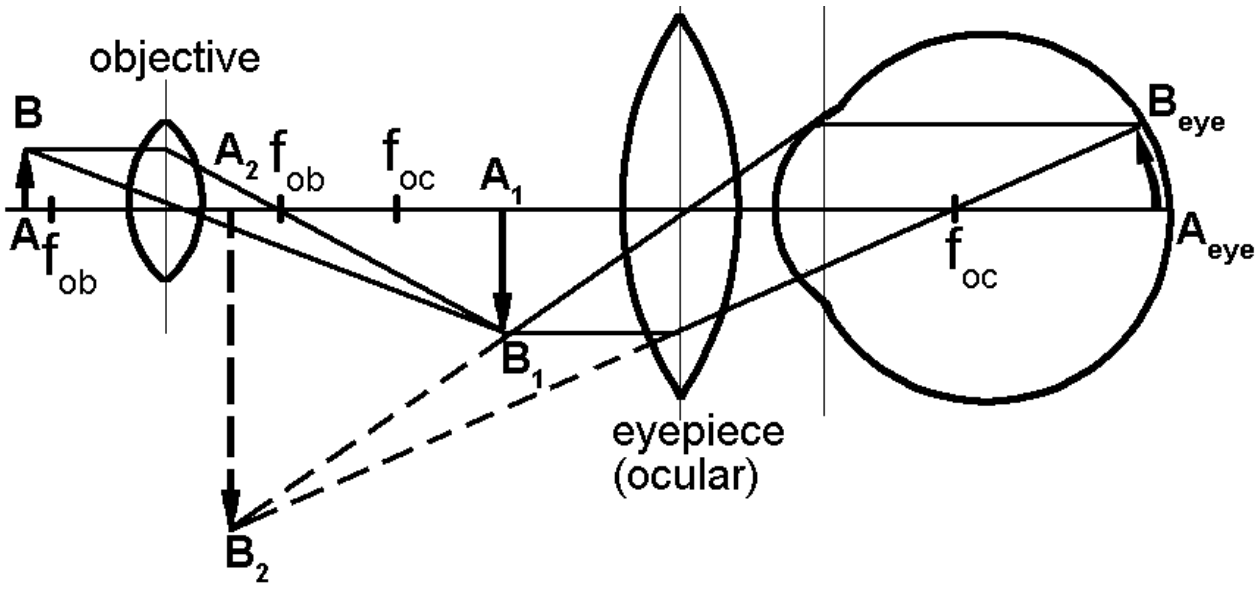

Fig.1. Microscope scheme.

The key part of a light microscope is its drawtube that encases two collapsible lens systems: the objective and the eyepiece. The path of the rays in the microscope is shown in Fig. 1.

The objective with a focal distance f_{ob} which is equal to several millimeters, and an eyepiece with a focal distance f_{oc} which is equal to several centimeters. Object AB places directly in front of focus f_{ob} of the objective. Its image A_1B_1 originates at the distance greater than $2f_{ob}$. As clear from the design, the objective creates a magnified, inverted, and real image.

The eyepiece is located thus that this image A_1B_1 was behind eyepiece focus f_{oc}.

The final image of the object A_2B_2 (studied in the microscope as a whole) is magnified, inverted, and virtual. In is located on the best vision distance (25 cm for a normal eye).

Total magnification of microscope V is product of separate magnifications of objective V_1 and eyepiece V_2:

$$V = V_1 \cdot V_2 = \frac{S \cdot l}{f f_1}, \quad (1)$$

where l is optical length of a tube (distance between focal points f_{ob} and f_{oc}), f_{ob} and f_{oc} are focal distances of the objective and an eyepiece accordingly, S is distance of the best sight.

Phenomenon of light diffraction limits maximum possible magnification. A resolving limit of a microscope (d) is the minimum distance between two points, which can be viewed in a microscope as two distinct points.

The formula for calculating the resolving limit of an optical microscope can be derived from the formulae, which describe the diffraction phenomenon. A resolving limit d is equal:

$$d = \frac{0{,}6\lambda}{n \sin\alpha}, \quad (2)$$

where λ – is wavelength of used light in vacuum, n – refraction index of medium between object and objective, α is the aperture angle – angle between ultimate rays of light beam, which left from object point and hit to the objective.

Wavelength λ is limited for visual light, therefore minimal d is equal approximately 0,2 mkm (it corresponds to maximal magnification nearly 1500 times.

Increase of the resolving limit can be achieved by diminution of wavelength. There are two main methods. For example, it can be using ultraviolet light and quartz lenses (transparent for ultraviolet radiation) in microscope. Common glass, which is used for

making light microscope lenses, is opaque to ultraviolet radiation. Obtained image must be photographed or transformed into visible form by luminescent screen or by image converter tubes.

Scanning Electron Microscope allow scientists to view a universe too small to be seen with a light microscope. SEMs don't use light waves; they use electrons (negatively charged electrical particles) to magnify objects up to two million times. In electron microscope for obtaining of image accelerated high-energy electrons beams are used. Due to dual particle-wave properties these electrones behave like very short wave light and allow to obtain images with nanometer size details. ***Transmission Electron Microscope*** – also uses electrons, but instead of scanning the surface (as with SEM's) electrons are passed through very thin specimens.

The image formed by the microscope during ordinary microscopy is virtual. A real image is formed on the retina because the eye, which has a sufficiently high optical force, collects diverging rays, which exit the eyepiece. If one attempts lo obtain an image on a screen rather than on the retina, he must make the image formed by the microscope real. It can be formed by increasing the distance between the objective and the eyepiece so that the image formed by the objective is located far from the eyepiece at a distance greater than the focal distance, rather than between the eyepiece and the focus, as in ordinary microscopy. If in so doing the image is formed on the screen, this is ***microprojection***; and if the image is formed on a photographic plate, this is ***photomicrography*** [microphotography].

Brightfield - This is the basic microscope configuration (the images seen thus far are all from brightfield microscopes). This technique has very little contrast; in the images you've seen so far, much of the contrast has been provided by staining the specimens.

Darkfield - This configuration enhances contrast.

Phase contrast - This technique is best for looking at living specimens, such as cultured cells.

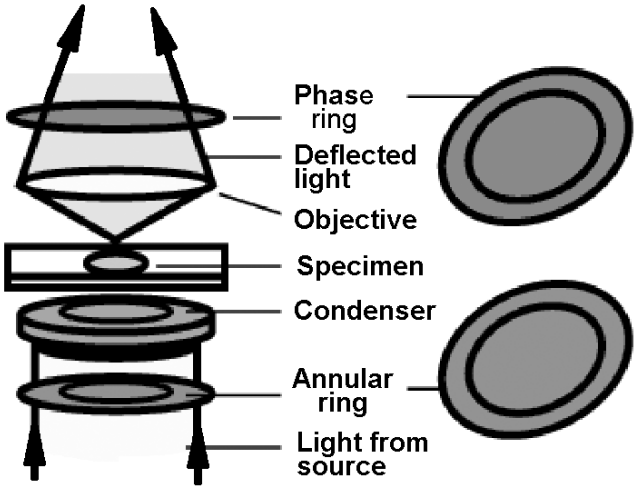

Fig.2 Phase-Contrast Light Pathways.

In a phase-contrast microscope, the annular rings in the objective lens and the condenser separate the light. The light that passes through the central part of the light path is recombined with the light that travels around the periphery of the specimen. The interference produced by these two paths produces images in which the dense structures appear darker than the background.

During incidence on a transparent object, a fraction of light passes through this object, and a fraction scatters on the object elements distinguished by the refraction coefficient. The rays passing through the object and those scattered differ in phase slightly, and when they meet on the screen, they interfere. Since their phase differences are small, in case of transparent objects, the rays create illumination on the screen, which differs little from that in adjacent points of the screen.

For augmentation of phase contrast of adjacent points special methods are used. At normal incidence of parallel light rays on the object, rays passed through object without scattering go directly pass through the objective focus. A phase plate, which increases the optical path of rays, is placed in the focus area. It can to increase illumination of the adjacent points as result of interference. It is necessary to use special objectives and special condensers.

The other way is using of phase plate with an aperture in its centre. In so doing, the optical length of the path changes to change the phase of rays scattered on the object, rather than that of rays, which have passed directly through the object.

Differential interference contrast (DIC) - DIC uses polarizing filters and prisms to separate and recombine the light paths, giving a 3-D appearance to the specimen.

Stereoscope - this microscope allows for binocular (two eyes) viewing of larger specimens. (The spinning microscope at the top of this page is a stereoscope).

Polarization - The polarized-light microscope uses two polarizers, one on either side of the specimen, positioned perpendicular to each other so that only light that passes through the specimen reaches the eyepiece. Light is polarized in one plane as it passes through the first filter and reaches the specimen. Regularly-spaced, patterned or crystalline portions of the specimen rotate the light that passes through. Some of this rotated light passes through the second polarizing filter, so these regularly spaced areas show up bright against a black background.

Fluorescence - This type of microscope uses high-energy, short-wavelength light (usually ultraviolet) to excite electrons within certain molecules inside a specimen, causing those electrons to shift to higher orbits. When they fall back to their original energy levels, they emit lower-energy, longer-wavelength light (usually in the visible spectrum), which forms the image.

The fluorescent molecules within the specimen can either occur naturally or be introduced. For example, you can stain cells with a dye called calcein/AM. By itself, this dye is not fluorescent. The AM portion of the molecule hides a portion of the calcein molecule that binds calcium, which is fluorescent. When you mix the calcein/AM with the solution bathing the cells, the dye crosses into the cell. Living cells have an enzyme that removes the AM portion, traps the calcein within the cell and allows the calcein to bind calcium so that it fluoresces green under ultraviolet light. Dead cells no longer have this enzyme. Therefore, living cells will fluoresce green, and dead cells will not fluoresce. You can see the dead cells in the same specimen if you mix in another dye called propidium iodide, which only penetrates the dead cells. Propidium iodide binds to DNA in the nucleus and fluoresces red under ultraviolet light. This double-dye technique is used in toxicology studies to determine the percent of a cell population that is killed when treated with some environmental chemical, such as a pesticide.

Fluorescence-microscopy techniques are useful for seeing structures and measuring physiological and biochemical events in living cells. Various fluorescent indicators are available to study many physiologically important chemicals such as DNA, calcium, magnesium, sodium, pH and enzymes. In addition, antibodies that are specific to various biological molecules can be chemically bound to fluorescent molecules and used to stain specific structures within cells.

Microscopy method

Devices and goods:

A light microscope, stage micrometer, tooth microsection, human hair, thin plant leaf, onion scales, blood smear.

Coarse focus = rough (crude) focusing = coarse adjustment.

Sample = specimen.

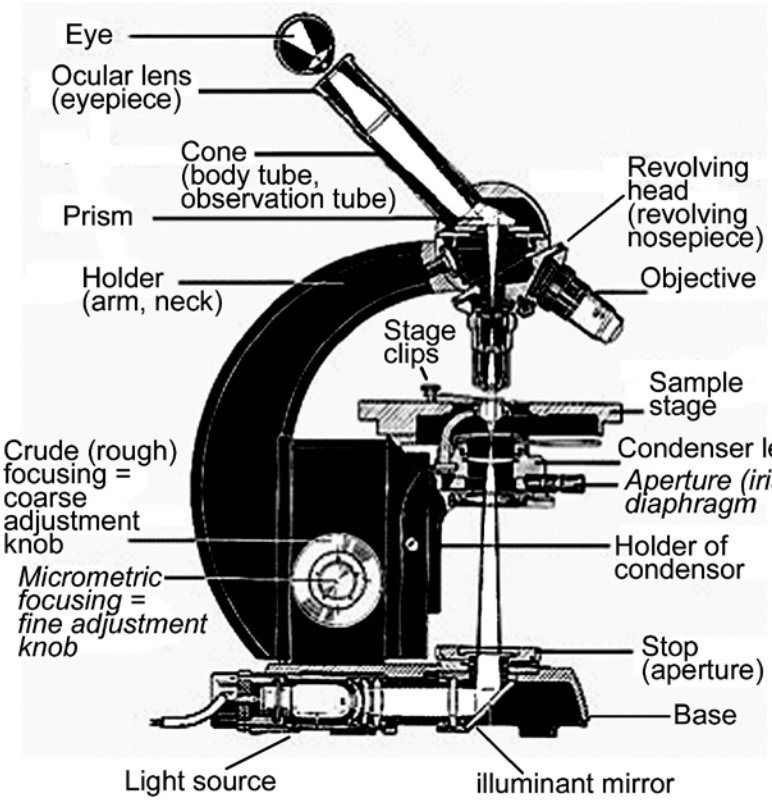

Fig.3. Optical microscope cross-section.

Modernt variants of microscopes are represented on follows pictures.

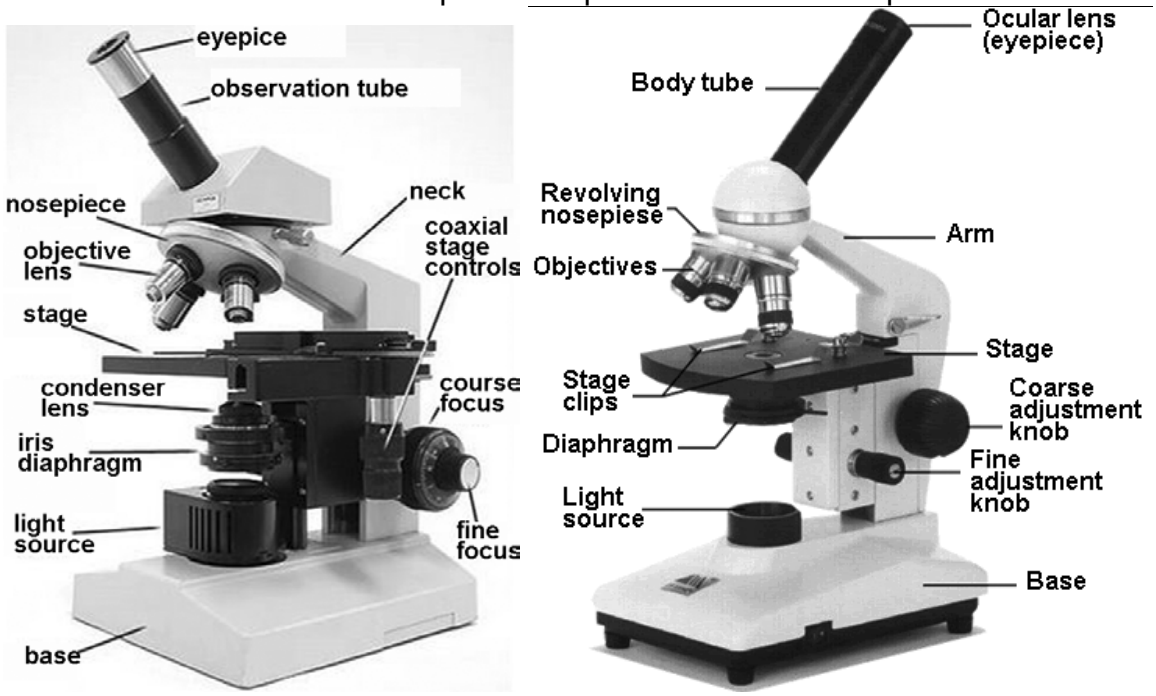

Fig.4. Optical microscope overview (variantes).

If stage has round shape with incisions over the entire side, it is mean, that stage can be rotated. In other case coaxial stage controls handle can be at side of stage.

Small handles on sides of the stage are control of stage sliding movement right–left and forward–backwards usually.

The parts of a light microscope

A light microscope, whether a simple student microscope or a complex research microscope, has the following basic systems:

– specimen control - hold and manipulate the specimen;

- stage - where the specimen rests;
- clips - used to hold the specimen still on the stage (Because you are looking at a magnified image, even the smallest movements of the specimen can move parts of the image out of your field of view.);
- micromanipulator - device that allows you to move the specimen in controlled, small increments along the x and y axes (useful for scanning a slide);
- Illumination - shed light on the specimen (The simplest illumination system is a mirror that reflects room light up through the specimen.);
- lamp - produces the light (Typically, lamps are tungsten-filament light bulbs. For specialized applications, mercury or xenon lamps may be used to produce ultraviolet light. Some microscopes even use lasers to scan the specimen.);
- rheostat - alters the current applied to the lamp to control the intensity of the light produced;
- condenser - lens system that aligns and focuses the light from the lamp onto the specimen;
- diaphragms or pinhole apertures - placed in the light path to alter the amount of light that reaches the condenser (for enhancing contrast in the image).

Lenses – form the image:

- objective lens - gathers light from the specimen;
- eyepiece - transmits and magnifies the image from the objective lens to your eye;
- nosepiece - rotating mount that holds many objective lenses;
- tube - holds the eyepiece at the proper distance from the objective lens and blocks out stray light.

Focus - position the objective lens at the proper distance from the specimen:

- coarse-focus knob - used to bring the object into the focal plane of the objective lens;
- fine-focus knob - used to make fine adjustments to focus the image.

Support and alignment:

- arm - curved portion that holds all of the optical parts at a fixed distance and aligns them;
- base - supports the weight of all of the microscope parts.

The tube is connected to the arm of the microscope by way of a rack and pinion gear. This system allows you to focus the image when changing lenses or observers and to move the lenses away from the stage when changing specimens.

Light microscopes can reveal the structures of living cells and tissues, as well as of non-living samples such as rocks and semiconductors. Microscopes can be simple or complex in design, and some can do more than one type of microscopy, each of which reveals slightly different information. The light microscope has greatly advanced our biomedical knowledge and continues to be a powerful tool for scientists.

Some Microscope Terms

- Depth of field - vertical distance, from above to below the focal plane, that yields an acceptable image.
- Field of view - area of the specimen that can be seen through the microscope with a given objective lens.
- Focal length - distance required for a lens to bring the light to a focus (usually measured in microns).
- Focal point/focus - point at which the light from a lens comes together.
- Magnification - product of the magnifying powers of the objective and eyepiece lenses.
- Numerical aperture - measure of the light-collecting ability of the lens.
- Resolution - the closest two objects can be before they're no longer detected as separate objects (usually measured in nanometers).

Magnification

Sign “x” marks magnification of lens.

Your microscope has 3 magnifications: Scanning (minimal), Middle and High. Each objective will have written the magnification. In addition to this, the ocular lens (eyepiece) has a magnification. The total magnification is the ocular magnification Ч objective magnification.

Dark stripe is used for oil immersion objective marking.

	Objective magnification	Ocular lens magnification	Total Magnification
Scanning (low power)	8x	7x	56x
Middle Power	40x	7x	280x
High Power (immersion objective use)	70x or 100x	7x	490x or 700x

Types of objective and ocular lenses used in USA and Britain have other magnifications and marking (next characteristics): the objective lenses, located on the rotary nosepiece, provide 4 different degrees of magnification (British notation):

Name	Characteristics	Magnifying power
Scanning power	shortest objective, red stripe	4x
Low power	next shortest, yellow stripe	10x
High-dry power	intermediate length, blue stripe	40, 43 or 45x
Oil immersion	longest, black stripe	100x

The ocular lens, located nearest to your eye, has a magnification power of 10x.The total magnification is determined by multiplying the power of the objective by the power of the ocular. (For example, 4x times 10x = 40x).

General Procedures

1. Always start and end with the Scanning Objective. Do not remove slides with the high power objective into place – this will scratch the lens!
2. Always wrap electric cords and cover microscopes before returning them to the cabinet. Microscopes should be stored with the Scanning Objective clicked into place.
3. Always remove the sample from the stage before microscope transportation.
4. Always carry microscopes by the holder (arm) and set them flat on your desk.

Focusing Specimens

1. Set optimal illumination of field of vision in eyepiece.
2. Always pull down microscope observation tube under observation from the side, do not look into the ocular. You can damage lense and slide (sample), as object-plates can have various thicknesses.
3. Always start with the scanning objective. Odds are, you will be able to see something on this setting. Use the Coarse Knob to focus, image may be small at this magnification, but you won't be able to find it on the higher powers without this first step. Do not use stage clips, try moving the slide around until you find something.
4. Once you've focused on Scanning, switch to Low Power. Use the Coarse Knob to refocus. Again, if you haven't focused on this level, you will not be able to move to the next level.
5. Now switch to High Power. (If you have a thick slide, or a slide without a cover, do NOT use the high power objective). At this point, ONLY use the Fine Adjustment Knob to focus specimens.
6. If the specimen is too light or too dark, try adjusting the diaphragm.
7. If you see a line in your viewing field, try twisting the eyepiece, the line should move. That's because its a pointer, and is useful for pointing out things to your lab partner or teacher.

Ending

1. Store microscopes with the scanning objective in place.
2. Wrap cords of light source and cover microscopes.

Image Quality

When you look at a specimen using a microscope, the quality of the image you see is assessed by the following:

Brightness - How light or dark is the image? Brightness is related to the illumination system and can be changed by changing the voltage to the lamp (rheostat) and adjusting the condenser and diaphragm/pinhole apertures. Brightness is also related to the numerical aperture of the objective lens (the larger the numerical aperture, the brighter the image).

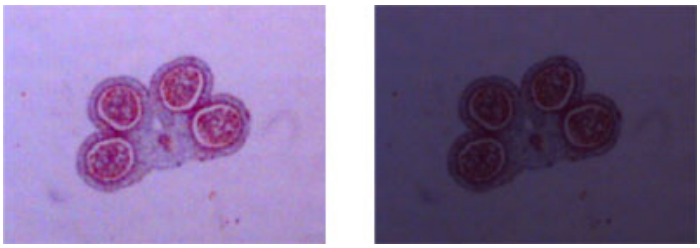

Fig.5. Image of pollen grain under good brightness (left) and poor brightness (right).

Focus - Is the image blurry or well-defined? Focus is related to focal length and can be controlled with the focus knobs. The thickness of the cover glass on the specimen slide can also affect your ability to focus the image -- it can be too thick for the objective lens. The correct cover-glass thickness is written on the side of the objective lens.

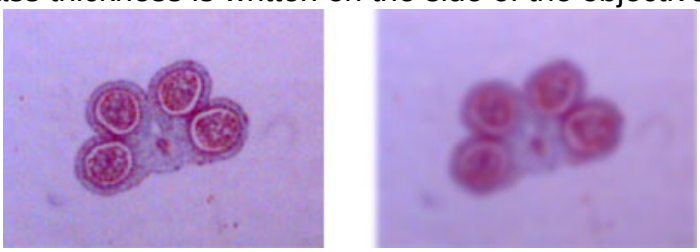

Fig.6. Image of pollen grain in focus (left) and out of focus (right).

Resolution - How close can two points in the image be before they are no longer seen as two separate points? Resolution is related to the numerical aperture of the objective lens (the higher the numerical aperture, the better the resolution) and the wavelength of light passing through the lens (the shorter the wavelength, the better the resolution).

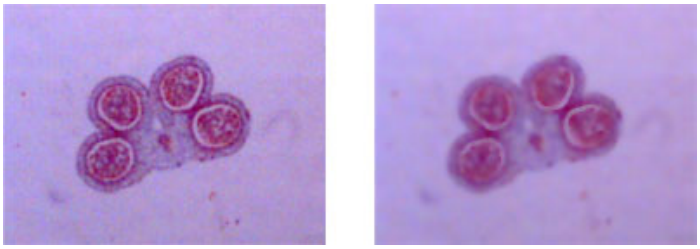

Fig.7. Image of pollen grain with good resolution (left) and poor resolution (right).

Contrast - What is the difference in lighting between adjacent areas of the specimen? Contrast is related to the illumination system and can be adjusted by changing the intensity of the light and the diaphragm/pinhole aperture. Also, chemical stains applied to the specimen can enhance contrast.

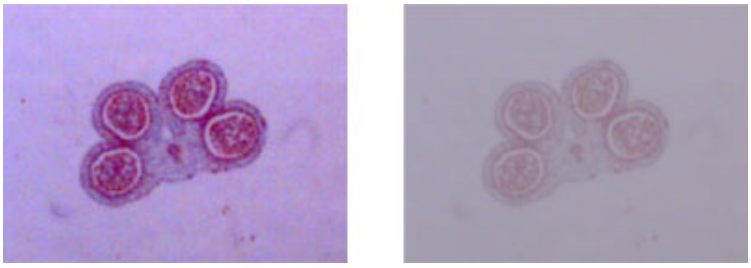

Fig.8. Image of pollen grain with good contrast (left) and poor contrast (right).

Depth of focus is interval of object details from the objective, in which object point are distinct. The more magnification, the less depth of focus (fig.9).

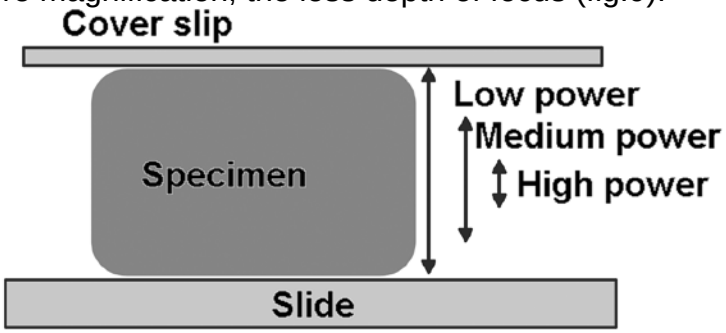

Fig.9. Depth of focus. Bidirectional arrows show layers, in which images are distinct.

Troubleshooting

Occasionally you may have trouble with working your microscope. Here are some common problems and solutions.

1. Image is too dark: Adjust the diaphragm (condenser), make sure your light is on.
2. I can't see anything under high power!

Remember the steps, if you can't focus under scanning and then low power, you won't be able to focus anything under high power.

3. Only half of my viewing field is lit, it looks like there's a half-moon in there!

You probably don't have your objective fully clicked into place.

Self-control material.

A. Questions to be answered:

1. Thin lenses kinds, light pathways in thin lenses; image forming pathways in thin lenses.
2. To know thin lens formula.
3. To be able draw converging and diverging lenses;
4. To be able draw images in converging and diverging lenses, when object is:
   a) between optical center and focus of lens;
   b) between focus and double focus of lens;
   c) after double focus of lens.
5. What is microscope? Describe purposes of its using in medical-biological research.
6. Name the key part of a light microscope and explain them functions.
7. To know rays pathes in microscope, creation of image by objective and eyepiece.
8. How to calculate microscope magnification? What factors influe on it? Give the definition and write formula of microscope magnification.
9. Give the definition and explain limitations and the resolving limit of a microscope. What is microscope resolution? Its maximal value.
10. What is numerical aperture? What is mean?
11. Give the definition and explain causes of aberrations appearance, methods of them reduction.
12. Give the definition and explain principles of special methods of microscopy: microphotographing and microfilming, binocular microscopy, stereoscopic (three-dimensional) binocular microscopy, ultramicroscopy, ultraviolet, dark-field,

luminecscent microscopy, polarizing microscopy, phase-contrast microscopy, microscopy with immersion.

13. Explain the preference of electron microscopv.
14. Explain the preference of polarization microscopy and luminescent microscopy.
15. Describe using of fiber optics in medicine.

Literature recommended

Main sources.

- Chaliy A.V. at all., Biological and medical physics. – A.V. Chaliy et all.– Ed.A.V. Chaliy. – Vinnitsia, Nova Knyha, 2013. – 480 pp.
- Korovina L.D. Biophysics with beginnings of mathematical analysis and statistics. Extended course of lectures. Vol.3. Optics. Quantum phenomena. – Poltava, 2018.– 128 p.

Additional textbook, journals and references::

- Compendium of Medical Physics, Medical Technology and Biophysics for students, physicians and researchers. Nico A.M. Schellart. – Department of Biomedical Engineering and Physics Academic Medical Center University of Amsterdam.– Amsterdam.– 2009 (electronic book).
- Roland Glaser. Biophysics: An Introduction.– 2010.
- Philip Nelson. Biological Physics (Updated Edition).– 2007.
- Paul Davidovits. Physics in Biology and Medicine, Third Edition (Complementary Science). – 2007.
- Bengt Nölting. Methods in Modern Biophysics.– 2009.

Methodical elaboration have prepared by senior lecturer, PhD biol.Sc. Korovina L.D.