

MICROSCOPY I: BRIGHT-FIELD

The microscope is a very important tool used by cell biologists. Proper use of the microscope can often mean the difference between success and failure in your laboratory work therefore, you should be certain that you fully understand how to use your microscope before you leave the laboratory.

The laws of optics are rigid; therefore, the optimal conditions for correct observation with the microscope are well defined, any departure from them leads to an inferior image. You will need to know and understand how to appropriately adjust the microscope. Do not adjust a knob without understanding the consequences, or you may spend a lot of time trying to readjust the microscope to the appropriate setting. Each time the microscope is used the following sequences of adjustments should be followed.

Rules for handling the microscope and slides

1. Use two hands when carrying a microscope, be gentle, these are sensitive and expensive instruments. Keep the microscope upright, oculars are removable and could fall out.
2. Clean or dry lenses with grit-free **lens paper** only. Booklets of it are on your table's tray. Never use anything but lens paper. Other papers, tissues, or cloth will scratch lenses.
3. Keep eyepiece(s) in the microscope at all times to keep dust out of the tube.
4. Do not unscrew or otherwise tamper with lenses unless instructed to do so.
5. At the start, do not look into the microscope. Begin by raising the stage as high as it can go by turning the coarse focal knob. You can now look through the ocular and focus on the specimen by turning the focus knob very slowly, in one direction.
6. To change the magnification, grasp the **ring** of the revolving nosepiece and rotate until the desired objective clicks into place. **DO NOT** use the objectives to turn the nose-piece.
7. Each time you complete work, particularly with oil immersion, clean the lenses with lens paper. Clean oil and other liquids from the stage and table as well.
8. When you have finished with the microscope:
 - The **lowest power** objective (it is also the shortest one) or none, should be returned to the position of use, before removing the slide.
 - The **slide** is removed from stage and **cleaned** by rinsing in ethanol, unless it is a prepared slide, then it should go back onto a slide tray.
 - The **lenses** and **stage** should be clean and dry. The **cord** loosely wrapped about the arm.
 - The **ocular** rotated so it faces the back of the scope. (As not hit the back wall of the cabinet when returned to the shelf.)

Interpreting Microscopy Images

The conventional compound microscope consists of a system of trans-illumination lenses, an objective lens and an ocular. Appropriate mechanical fixtures facilitate focusing and

mounting specimens. The illumination system is designed to fill both the aperture and the field of the objectives. Objectives are designed to produce a real image of the specimen, imposed by the wave nature of light. Oculars are designed to produce (a) a virtual image, which can be observed visually, or (b) a real image which can be projected on a screen or photographic plate. Efficient use of the microscope and the interpretation of images will require consideration of the following factors:

1. Magnification: The bright-field microscope magnifies or enlarges an object so that it is visible to the observer. Magnification is achieved using a two-lens system, composed of the ocular lens and the objective lens. Most bright-field microscopes are equipped with three objective lenses with magnifying powers of 10X (low power), 40X (high power), and 100X (oil immersion). The objective lens is closer to the specimen and magnifies it, producing the “real” image. The real image is then projected up the body tube into the oculars, which further magnify the specimen by 10X to produce the final image. The total magnification achieved is determined by multiplying the magnifying power of the objective and the ocular lenses. Select a magnification such that the finest details of the image (determined by the resolving power of the microscope) can be resolved by the retina or photographic plate. Magnification, which exceeds this, is known as empty magnification since it reveals no additional detail. Magnification power is indicated on each lens.

2. Resolving power: It is not possible to achieve unlimited magnification in a light microscope because lenses are limited in their ability to distinguish two points that are very close. This property, which is called the resolving power, is defined as the least distance between points in the object that can be distinguished in the image¹⁾. The resolution achieved by light microscopes is on the order of 0.2 to 0.3 μm. Even a microscope utilizing UV light (λ = 230 nm) does not appreciably increase resolution.

3. Depth of field is the thickness of the specimen that is in sharp focus for any one set of focus conditions. As the aperture angle increases, the depth of field decreases to a limiting value of about 0.5 μm with the oil immersion objective. This depth, which is a small fraction of the thickness of a typical biological specimen, makes possible the “optical sectioning” of specimens.

$$d = \frac{0.6 \lambda}{n \sin \theta}$$

¹⁾ For a perfect lens with a circular aperture, the resolving power is given by: $d = \frac{0.6 \lambda}{n \sin \theta}$
where:

0.6 = constant related to the distance between two objects distinguishable by the human eye as separate entities.

λ = wavelength of light

n = refractive index of the medium through which the light travels

θ = 1/2 angle of the cone of light entering the objective lens

As **d** decreases, resolving power increases. This equation for resolving power applies to any kind of lens (telescope, camera, microscope, eye, etc.), but it assumes certain conditions of illumination that are not automatically met when a lens is used as a microscope objective. To satisfy these conditions the cone of light illuminating the specimen must equal the cone of light accepted by the lens. Thus there are three ways to improve the resolving power of a light microscope: (1) increase **n** (e.g., use oil immersion)

(2) increase **θ** (θ has been maximized to 90⁰ in our best microscopes, so that sin θ = 1)

(3) decrease the **wavelength of light** (not too useful if the microscope depends on visible light for which λ is between 380 nm and 750 nm).

4. Contrast depends on the signal-to-noise ratio and upon the physiology of vision. In this case the signal is the image of interest and the noise is background of light associated with out-of-focus images, light scattered by dirt on optical surfaces, etc. Detectable contrast depends upon the ability of the eye (or other measuring device) to make fine distinction in intensity and color on resolvable areas of the image. In general the greater the noise, the greater the signal intensity required to produce a detectable image. Most histological and cytological techniques have been

developed to compensate for the fact that cells in visible light display little contrast. Staining is a classic example of such techniques, both live and dead cell staining.

Typical compound light microscope

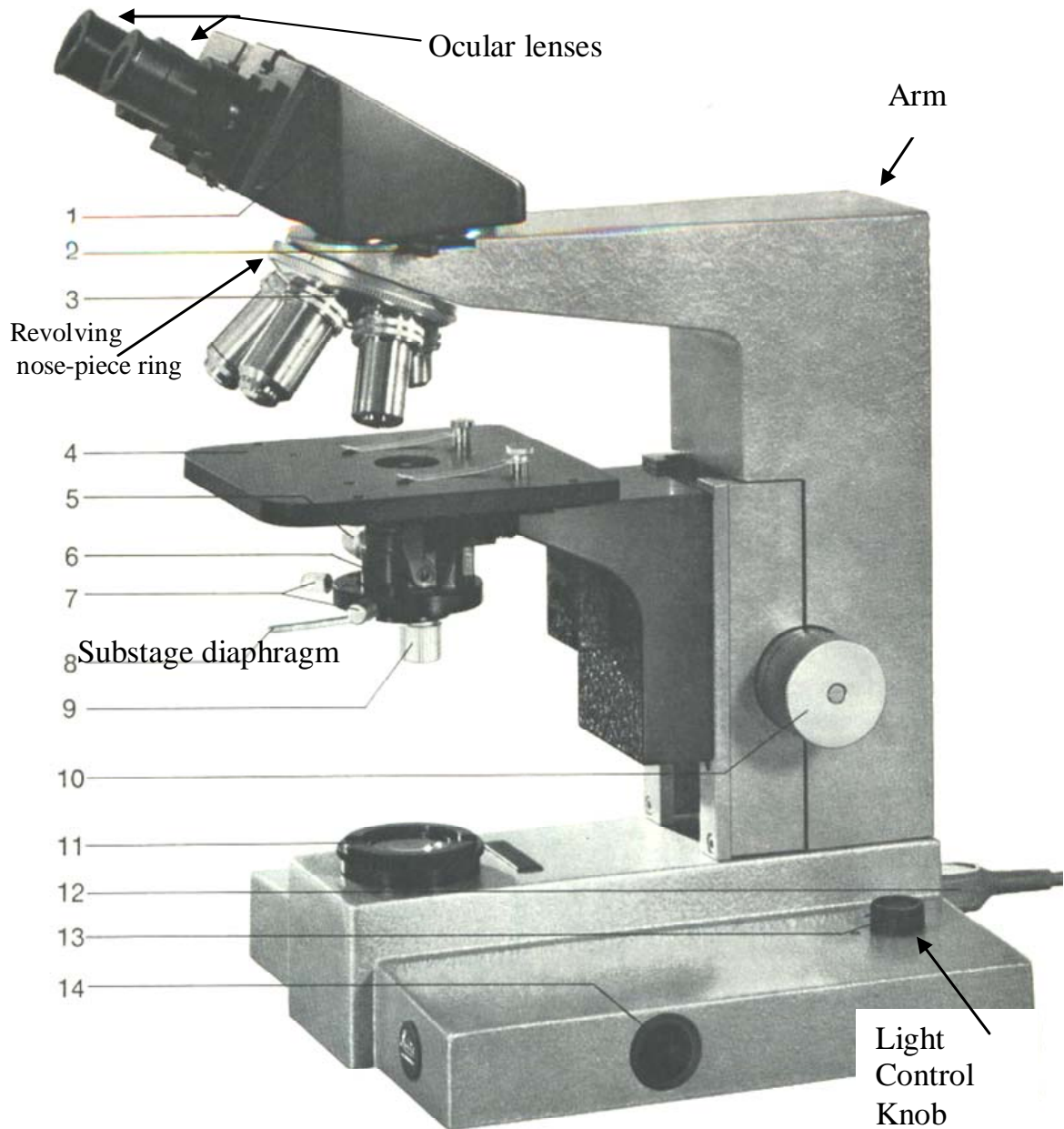


Fig. 1:
SM-LUX with binocular tube S and object stage No. 24a, with permanently mounted condenser

- 1 Binocular tube S
- 2 Tube changing lever
- 3 Revolving nosepiece with objectives
- 4 Object stage No. 24a with permanently mounted condenser
- 5 Lever for swing-out lens of condenser

- 6 Condenser
- 7 Centring screws
- 8 Aperture-stop lever
- 9 Control knob for vertical adjustment of condenser
- 10 Single control knob for coarse and fine focusing
- 11 Protective glass with field diaphragm
- 12 Mains cable
- 13 Control knob for transformer
- 14 Lamp socket

I. Alignment of the compound microscope for bright-field (Koehler) illumination.

Use of the microscope demands a precise alignment of all optical components.

- ❑ 1. Half close the lamp field diaphragm (above the lamp on the base of the scope), and open the **sub-stage diaphragm** all the way (on the condenser). Raise the condenser until it nearly touches the slide.
- ❑ 2. Carefully focus the 10X objective upon a prepared slide. Close the **sub-stage diaphragm** and note if the light is centered, if not tell your TA, it can be adjusted using two knobs on the condenser. Lower the **condenser** until a sharp image of the light source is obtained (blue halo). Open the sub-stage diaphragm. Check that the specimen is still in focus, adjust if not. Open the lamp iris until its aperture just matches the field of view. Slowly move the condenser until the light from the lamp is in focus.
- ❑ 3. Now remove an ocular and peer down the central axis of the microscope body tube. Note that when the **condenser iris** is closed, only a small central portion of the objective is receiving light. As you open the condenser iris you will see that the illuminated portion of the objective is bounded by an image of the condenser iris.
- ❑ 4. Adjust the light intensity for comfortable viewing, with the light control knob.
- ❑ 5. The microscope is now properly illuminated and adjusted for work with the 10X objective. When various oculars are used with this same objective lens, the only change that need be made is the stopping-down of the lamp iris until its sharply focused margin just coincides with that of the fields of view.
- ❑ 5. Change from the 10X objective to the 40X objective by rotating the nose-piece until the 40X objective clicks into place. Note that this lens is very nearly in focus (hence the nosepiece is said to be parfocal; if any one lens is focused upon an object, then any other lens rotated into place will either be in focus or very nearly so). Focus the lens.
- ❑ 6. With the ocular still out of the microscope, adjust the iris of the condenser so that the back lens is approximately matched by the aperture of the condenser iris. Replace the ocular: note the illumination of the field. Adjust lamp iris if necessary.
- ❑ 7. Rotate the nosepiece so that the oil immersion lens is coming into place, but stop before it clicks into place. With the glass rod of the immersion oil bottle, place a small drop (ca. 2mm in diameter) of immersion oil directly onto the coverslip above the point upon which the condenser beam is focused.
- ❑ 8. Rotate the nosepiece ring so that the immersion objective clicks into place. The space between its front lens and the cover slip is now filled with immersion oil of the same refractive index as glass. Focus lens.
- ❑ 9. Examine the field. Alter the adjustment of the lamp iris if necessary. The microscope is now properly illuminated and ready for work. The **FINE ADJUSTMENT ONLY** should be used when working with the oil immersion lenses.

II. How to measure images in the light microscope.

(Alexey Veraksa)

How would you go about measuring the diameter of a chloroplast, or the size of a Paramecium while you are looking under the microscope? A combination of two rulers (called micrometers) etched on glass are used: one is mounted on a regular slide and is placed on the stage (hence the name, **stage micrometer**), and another is placed in the microscope's eyepiece – this is an **ocular micrometer**. Two of them are needed because in the microscope, you normally cannot have both your specimen and a ruler next to it at the same time. Ocular micrometer is already installed in one eyepiece of your microscope. Remember that 1 mm (millimeter) is equal to 1000 μm (micrometers).

Fig. 1. Stage micrometer at different magnifications

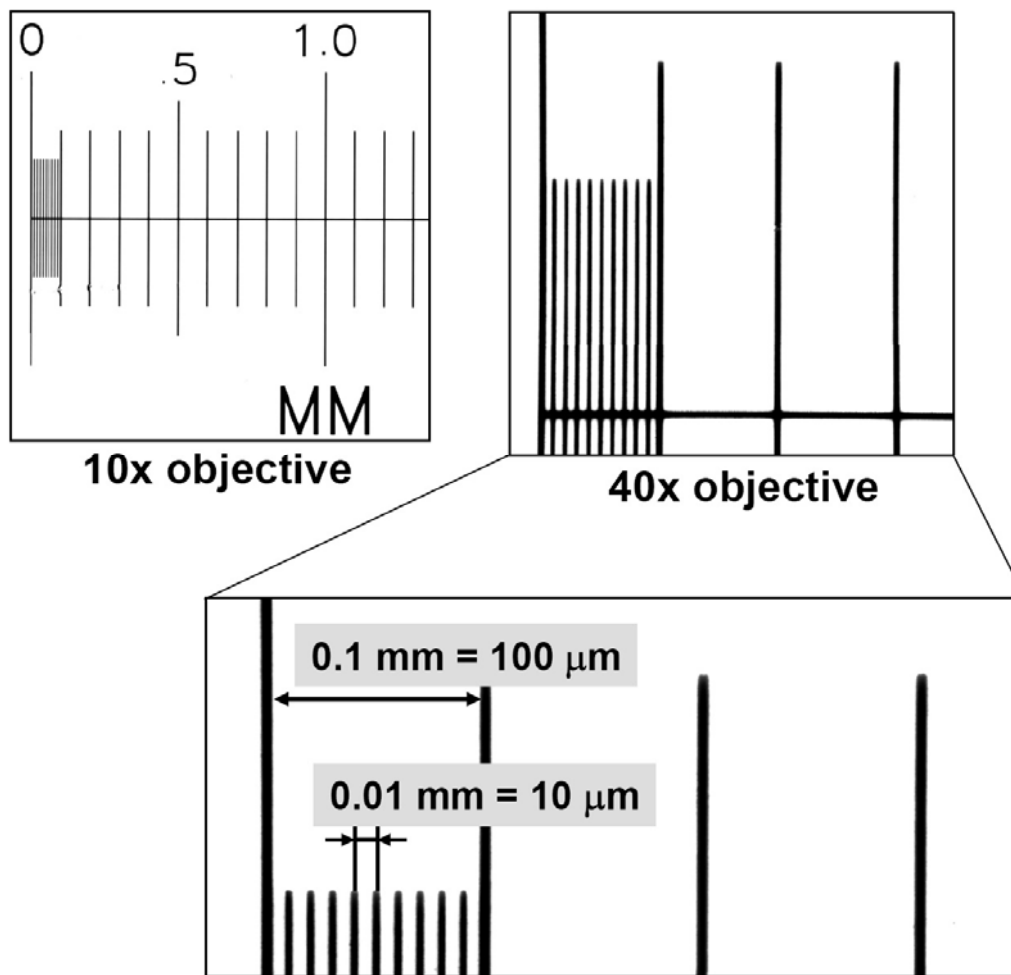
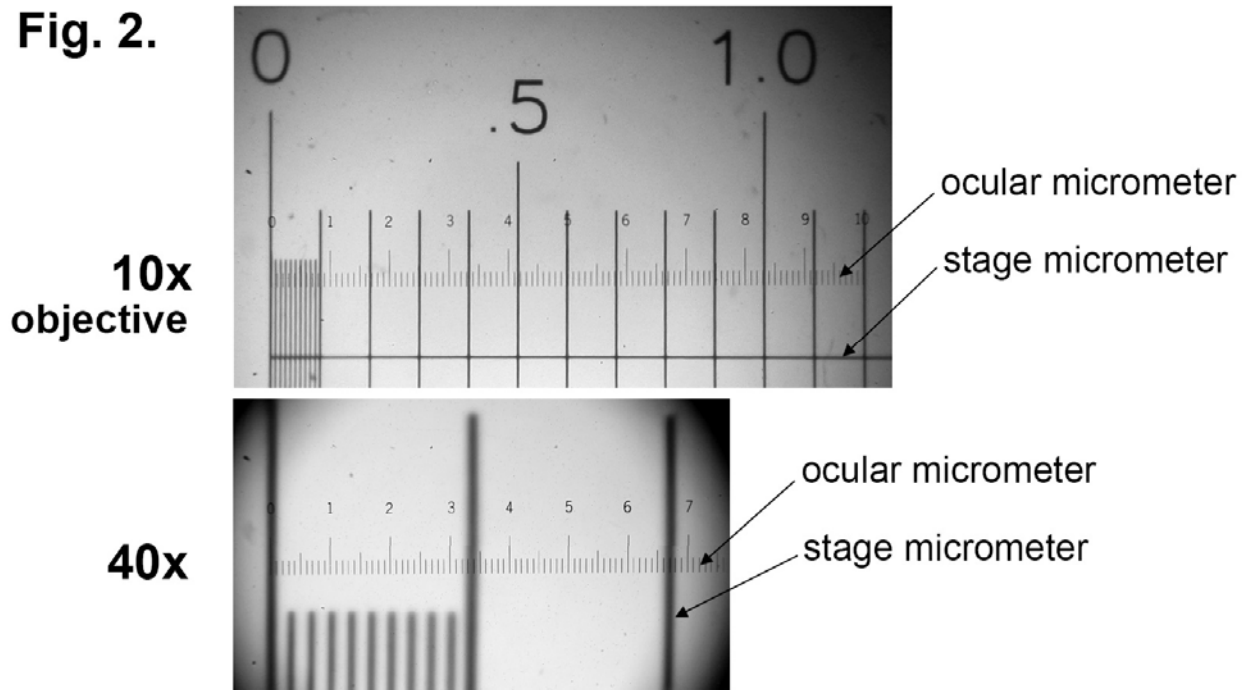


Fig. 1 depicts a stage micrometer at two different magnifications. Spend some time looking at the stage micrometer using different objectives and learn the relative sizes of the subdivisions. The smallest subdivision is 0.01 mm (10 μm), and the next one in size is 0.1 mm (100 μm). Note that as the power increases, less and less of the stage micrometer can fit in the field of view. Using the 100x objective, you can only see about 100 μm of your specimen, while using 10x you

can see over 1 mm. Magnification of the ocular micrometer doesn't change, however, regardless of what objective you use (see Fig. 2, where the stage micrometer is being visualized using either the 10x objective on top photo or the 40x objective in the lower photo.). In order to measure the size of an unknown object on the stage, first you have to calibrate the scale of the ocular micrometer using the stage micrometer, then remove the stage micrometer, place your specimen and measure it using the ocular micrometer. As you can readily see from Fig. 2, this process has to be performed independently for each objective used.

Fig. 2.



Here is a step-by-step procedure to measure an object on your slide:

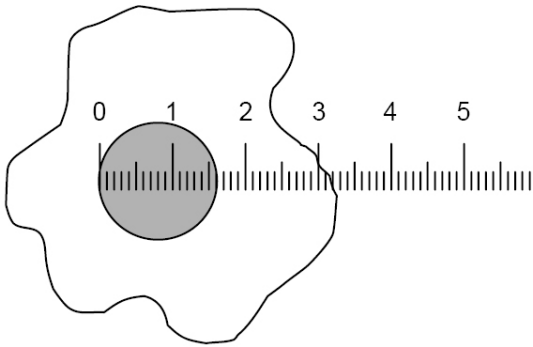
1. Choose an objective to start, e.g. 10x. (Again, note that this will need to be repeated for each objective under which you wish to measure an object.)
2. Place a slide with the stage micrometer on the microscope stage and focus on the ruler.
3. Make ocular micrometer parallel with the stage micrometer by turning the eyepiece. Align the 0 marks on the stage and ocular micrometers, as shown in Fig. 2.
4. Now you have to find out the scale of the ocular micrometer for that particular objective. Choose a subdivision of the *stage* micrometer that would span most of the length of the ocular micrometer. For example, in the 10x objective picture in Fig. 2, you can choose the 1.0 mm mark. In this example, it takes 83 subdivisions of the ocular micrometer to cover 1 mm of the stage micrometer (note that on the ocular micrometer scale, number 8 really means 80 small subdivisions). **For your measurements, you have to find the size of one subdivision of the ocular micrometer.** In order to do that, we divide 1 mm (which is equal to 1000 μm) by 83 subdivisions, and get approximately 12 μm per subdivision of the ocular micrometer. Write down this number and the corresponding objective. Note that you would arrive at the same number if you chose the 0.6 mm subdivision of the stage micrometer (which is 600 μm). You

would then divide it by 50, which is the marking of the ocular micrometer it overlaps with, to obtain the same 12 μm per one subdivision of the ocular micrometer.

5. Now it is time to measure an object. Remove the slide with the stage micrometer, place a slide with your specimen on the microscope stage and focus on it. For example, you want to measure the nucleus of a cell shown in Fig. 3. Align the 0 mark on the ocular micrometer with the left edge of your object (in our case, the left side of the nucleus). Read the length of the object from the scale – in our case, it will span 16 subdivisions of the ocular micrometer. If the object is not positioned correctly relative to the ocular micrometer (say, you want to measure the thickness of a hair but it runs horizontally on your slide), you can *turn the eyepiece* to properly align the ocular micrometer with the object you are measuring.

6. Multiply the number of subdivisions from the measurement by the length of one subdivision that you found in step 4. In our case, it will be 16 subdivisions \times 12 $\mu\text{m}/\text{subdivision} = 192 \mu\text{m}$. You have now found the exact size of this cell's nucleus.

Fig. 3.



In the 40x example shown in Fig. 2, the best way to find the scale of the ocular micrometer would be to choose the large subdivision of the stage micrometer that spans most of the field of view, which corresponds to 200 μm (consult Fig. 1 for stage micrometer markings). This line overlaps with the 67 mark on the ocular micrometer. Therefore, the scale of one subdivision of the ocular micrometer *for the 40x objective* would be 200 $\mu\text{m} / 67$ subdivisions = 3 $\mu\text{m} /$ subdivision. Given these calculations, if you now assume that the cell in Fig. 3 is observed using a 40x objective, what would be the size of its nucleus in μm ?

III. Observations:

During the remaining time, become familiar and comfortable with the microscope by focusing the prepared slides of various cells and tissues that are available in the lab. Practice measuring those items that you focus under the microscope, as this is an important skill.

1. Refer to the procedure instructions which follow in order to examine the prepared slides using various magnifications; sketch and label the cells and subcellular components. Record the cell type and the total magnification used; perform several measurements using the ocular and stage micrometers.

2. Refer to the procedure instructions which follow in order to prepare your own slides of cheek cells, onion cells and mixed plankton or protists. Make measurements using the ocular and stage micrometers.

PHASE-CONTRAST MICROSCOPY

In 1932 Fritz Zernicke invented the phase-contrast microscope, giving biologists their first look at the activities of living cells. The phase-contrast microscope is a light microscope outfitted with a special kind of optical system designed to exploit the diffracting properties of cells. Diffraction of light generates contrast and renders unstained cells visible. The phase-contrast microscope is used to view phase objects, which are simply objects composed of material with a refractive index unlike the medium that surrounds them.

In phase-contrast microscopy, light must be considered as waves, rather than photons. Light is emitted from a source either at a characteristic frequency (monochromatic light), or at a series of frequencies (polychromatic light). The frequency (f) is invariant, but as the light travels through various media of different refractive index, for example air, water, or cytoplasm, its velocity (v) and wavelength (λ) vary reciprocally:

$$f = \frac{v}{\lambda}$$

As light waves enter a medium from the air, they are slowed down, or retarded. On reemerging the light waves assume their original velocity.

Since formed elements in living cells, such as chromosomes, generally have a higher refractive index than the surrounding cytoplasm, light passing through them is affected in two closely related ways: (1) the phase of the light wave is retarded, the light travels at a lesser velocity through materials of higher refractive index, and (2) the direction of the light is changed and hence scattered outside of the phase disc in the objective. Elements of lower refractive index also scatter light but advance the phase of the light wave. With phase objectives of conventional design, structures of higher refractive index appear darker, while structures of lower refractive index appear lighter than the background. In order to achieve phase-contrast, two special parts are added to the conventional compound microscope (see Fig. 2.1) :

- 1.) a doughnut-shaped phase disc on the back of the objective and
- 2.) a doughnut -shaped stop (phase annulus) placed in the condenser.

Alignment of the phase microscope requires, in addition to the steps outlined in the section on the compound microscope, the centering of these two doughnut-shaped elements. When a specimen is moved into the field the image of the annulus will become fuzzy and a substantial amount of light will be scattered into other regions of the objective aperture. It is this scattered light which does not pass through the region of the objective aperture containing the phase disc that is responsible for producing the phase image of the specimen. The scattered light interferes with the unscattered light that has passed through the phase disc thus producing the differences in intensity.

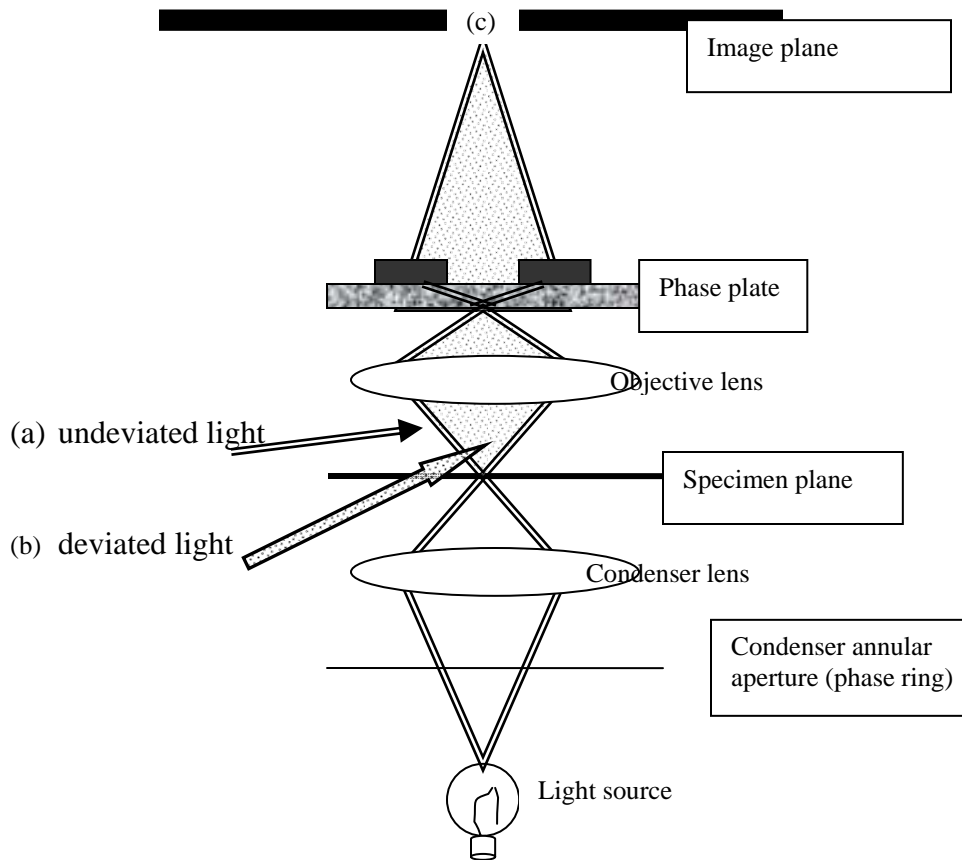


Figure 2.1: Optics of a phase-contrast microscope. Its phase is changed by going through the phase plate. (b) The deviated light is the light which goes through the object. Its phase is changed by going through different parts of the object. (c) The two types of light overlap at the image plane, where either amplification of the wave or destructive interference may take place, depending on the type of microscope used.

Procedure:

1. Place a prepared slide with a stained specimen on the stage of the phase-contrast microscope, using Bright-field (H) and focus the image. Note the appearance of the specimen.
2. Examine the stained slide a second time but using phase-contrast instead of bright field (condenser set at 1 for 10X, 2 for 40X), compare to the observations made above.
3. Make a “cheek cell” preparation on a labeled slide. (unstained followed by stained)
 - Put a drop of water on a slide. Using a tooth pick, gently swab the inside of your cheek. This contains squamous epithelial cells; mix it in the water on the slide.
 - Cover the preparation with a coverslip. Toss the toothpick into the trash.
 - Examine the slide using the bright-field microscope condenser setting H. Regulate the illumination for good contrast. Note the bright-field image, of the cell surface.
 - Examine the same slide a second time but using phase-contrast microscope condenser settings, set at 1 for 10X, and 2 for 40X. Compare the images to those with bright-field.
4. Make a stained “cheek-cell” preparation on a labeled slide.
 - Place a drop of Trypan Blue stain on a clean slide. Using a tooth pick, gently swab the inside of your cheek. Mix it in the drop of stain on the slide and gently stir the two together.

- Cover the preparation with a coverslip.
- Examine the stained slide using the bright-field microscope (condenser set at H).
- Remember to regulate the illumination to get good contrast. Note the bright-field image, looking closely at the cell surface.
- Examine the stained slide a second time but using phase-contrast instead of bright field (condenser set at 1 for 10X, 2 for 40X), making the same observations as above. Compare the images seen.

Compare the stained cells to the unstained cells viewed by phase-contrast microscopy.

Determine the average width of a cheek cell by measuring the width of three or more cells.

On a drawing of an individual stained cell, label the following components:

Nucleus (spherical darkly stained body near the center of the cell)

Chromatin (stained thread-like material within the nucleus)

Nucleolus (tiny darker stained body within the nucleus)

Cytoplasm (lightly stained fluid within the cell membrane)

Cytoplasmic organelles & inclusions (small, often darkly stained bodies in the cytoplasm)

Plasma membrane (boundary of the cell)

5. Place a drop of iodine or methylene blue stain on a clean slide. Use forceps to strip the transparent “skin” from the inner surface of an onion and spread the skin in the stain; apply a coverslip. Examine with low and high power by phase-contrast microscopy; identify the following cell structures: **Nucleus** **Vacuole** **Cytoplasm** **Cell wall**

Determine the average width of an onion skin cell by measuring the width of three or more cells and compare it to the size of the average cheek cell.

6. Place a drop of pond water on a clean slide. Examine the unstained preparation. Fix the specimen by adding 2-3 drops of 1% acidulated methyl green, mix gently and cover. Examine, measure, and sketch a few of the microorganisms using phase-contrast microscopy.

7. Choose a large object from the pond or salt water mix to focus on. Recall that three-dimensional objects have depth as well as length and width. With a thick specimen you will have to focus up and down (optical sectioning) to observe the cell’s depth.