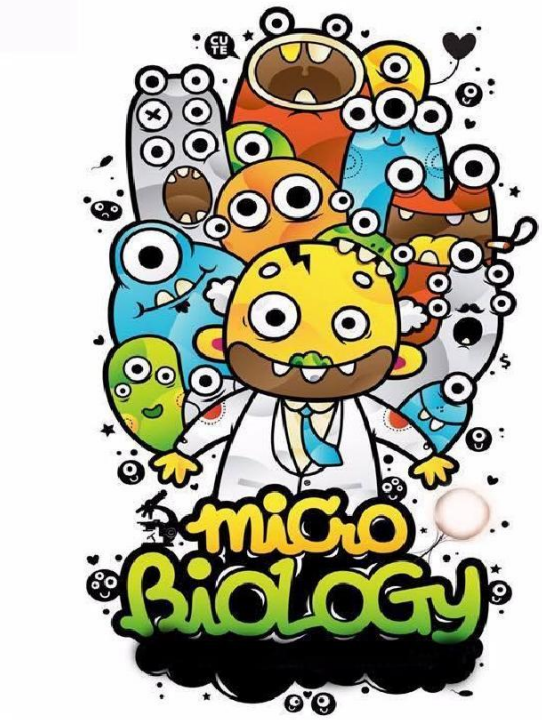


Subject:	Microscopy
Done by :	Fahed AlKarmi
Corrected by:	Karam Darwish
Lecture number:	1

Microscopy

Microbiology is the study of microorganisms, and the microbiology didn't develop until the invention of the microscope because the microorganisms are very very small and can't be seen by the naked eye. We use the microscope to study the shape, division and motility of the microorganisms (most of them are unicellular) so we must view the cells under the microscope to study these aspects.

The size range of most bacteria is 0.5-2 µm and this range can't be seen with the naked eye. We must differentiate between seeing something and distinguishing something, for example, I can see the table and everything on it but I can't distinguish the bacteria on it, things with a size less than 0.1 mm can't be distinguished with the naked eye, and most human cells and all microorganisms are smaller than 0.1 mm in size, so we use microscopes to see and distinguish them.

A) Light microscope (LM)

We can't view single atoms under the LM but we can distinguish things with size more than 0.2 µm so we can view prokaryotic cells like bacteria, eukaryotic cells and some of their organelles like the golgi apparatus and the mitochondria which have the same size as bacteria, but we can't view viruses under the LM because their sizes are less than 0.2 µm so we use the electron microscope here, also we can't view some subcellular organelles like ribosomes under the LM. Electron microscopes have a better resolution than LM, that's why we use them to view viruses and the ultrastructure of the cells, and resolution is the ability to distinguish two objects as separated two objects, and when the objects are smaller or closer to each other a higher resolution (resolving power) is needed. The term "light microscope" is a general term for microscopes that use light as a media of visualization and we have many types of LM and the compound LM (brightfield microscope) is the most common one and we will mention 4 major types of LM with their structure and mechanism.

1- Brightfield microscope (compound LM)

Here, the background (field) is brighter than the sample hence its name is brightfield microscope.

Structure: It is the simplest type despite its other name “compound LM”, it has a light source (usually it is white -mix of many wavelengths-), after that it has a condenser to condense (focus) the light on the specimen upon the adjustable stage, then the light passes through the specimen to reach the objective lens which collects the light and magnifies the image of the specimen (first magnification) and then the collected light reaches the ocular lenses for a second magnification, the overall magnification = the first magnification (objective lens) x the second magnification (ocular lenses).

Mechanism: The light source emits light with a specific intensity and passes through the specimen, if it passes through the background nothing will happen (the intensity and the color of it stay the same), but when the light passes through the sample it will be either absorped or scattered so the the intensity of the transmitted light will decrease after passing through the sample, this decrease or reducuction in the intensity is called “attenuation” and caused by the absorption and the scattering of the light, without attenuation we can’t distinguish between the sample and the background which is brighter than the sample. The contrast in this microscope comes from the difference in the intensity and also the color between the sample and the background, and between different spots on one sample, because materials absorb certain wavelengths and allow others to pass. for example all light colors can be absorped by the sample except blue, so we see it blue . Unfortunately, most cells are transparent so there is no attenuation, so how can we distinguish them? We use stains in this type of microscopes to increase the contrast between the sample and the background.

Disadvantages: we can’t view things with size less than 0.2 µm. Moreover, stains kill the cells and that prevents us from observing the motility and the division of the cells.

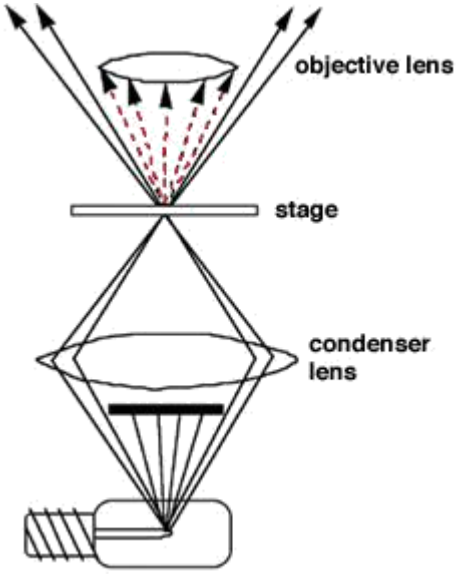
2-Darkfield microscope

Here, the background (field) is darker than the sample hence its name is darkfield microscope.

Stains aren't used in this type of microscopes so cells are still alive, and because of the absence of stains here, the mechanism must be different.

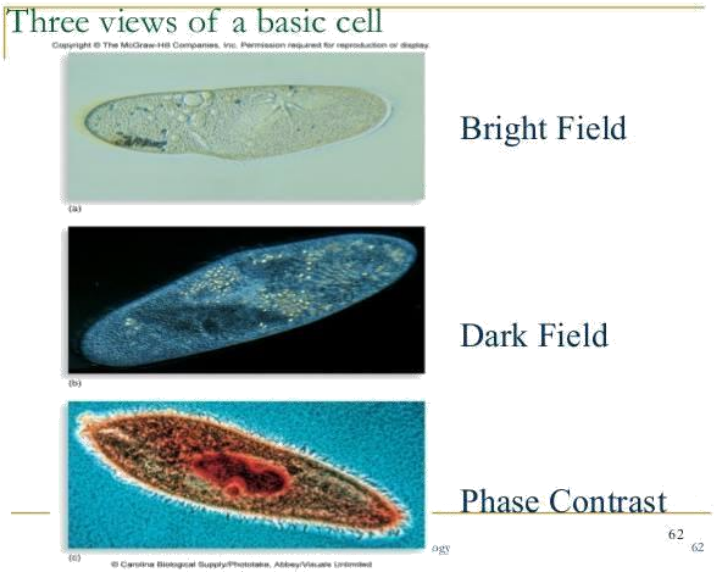
The structure of this microscope is almost like the structure of brightfield microscope, there is just a small difference which is the condenser.

The condenser has a special diaphragm which prevents the direct transmission of the light through the sample into the objective lens by changing the angle of the light which passes through the sample and transmits to the outside of the stage so the background appears darker because it doesn't reflect the light. The reflected light from the sample only will be collected by the objective lens (see the figure below). If the sample can't absorb, scatter or reflect the light then neither brightfield microscope nor darkfield microscope could be used, in this case we use the third type which is phase contrast microscope.



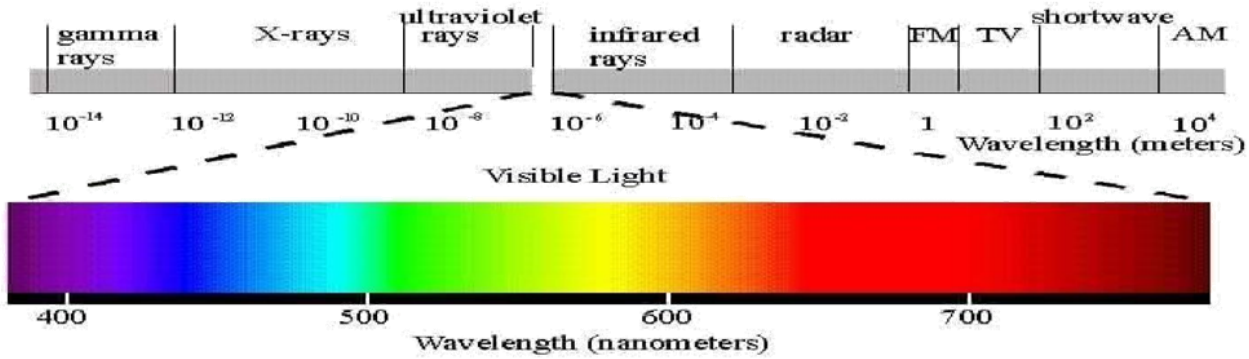
3-Phase contrast microscope

The first type depends on the ability of the sample to absorb and scatter light, the second type depends on the ability of the sample to scatter (reflect) the light. This type depends on the diffraction of light rays when they pass through an object and they change their direction not their speed, and by changing the direction of light it will reach the objective lens a bit later than light rays that pass through background because the sample makes the light cross longer distance, this lateness is called phase shift, note that the intensity and the brightness aren't changed here, this phase shift can't be detected by our eyes so we change the condenser and the objective lens which –in addition to magnifying the image- converts the phase shift into brightness difference, these specially designed objective lens decreases the intensity for the late light rays and appears darker, that makes sense because our eyes can detect the brightness difference (the physics of it is very complicated and we only need to know the general principle). Note that no stains are used here so we can view cell activities and motility. The last 3 microscopes could be in a 3in1 compound microscope (the condenser and the objective lens are changeable) which is more expensive than a single function microscope.



4-Fluorescent microscope

It is a special type of LM which uses fluorescence. Fluorescence is a phenomenon in which light is emitted. The phenomenon of light emission is called luminescence which has many types. light is a form of energy and energy can neither be created nor destroyed; rather, it transforms from one form to another, so objects need to absorb energy in order to emit light and this energy comes from different sources and depending on the source of energy we subdivide luminescence into different types, for example, exothermic reactions give energy and this energy could be in the form of ATP , heat and light, and when light is emitted due to chemical reaction, this is called chemoluminescence. When the energy source which leads to light emission is light, that is called **fluorescence** and it has special conditions. Every electron has a ground state, and when an electron absorbs the energy of a specific light (could be UV or visible) it transforms to the excitation state (higher energy level), the excited electron tends to go back to the ground state (relaxation state) , that is not happening until it losses energy, the losing of energy happens in more than one step to reach the ground state, the lost energy is in the form of light and it is called fluorescence **only** if the emitted light is in the visible range . a fluorophore is a fluorescent chemical compound. The energy of the emitted light is less than the energy of the excitation light because it is emitted in more than one step (it could be emitted in one step but this is rare) and the wave length is higher than the excitation light (Wavelength and Energy are inversely proportional). for example if a dye absorbs blue light it will emit green light, and when we say “a green dye” this means that the emitted light is green and the excitation light has higher energy than the energy of green light, if the dye is red then the excitation light is green and if the dye is blue (like DAPI blue which is famous in medicine) the excitation light is UV.(so we name the dye according to the color of the emission light not the excitation light)
*Note: for most fluorescent dyes, the excitation and emission are characteristic for each fluorescent substance.



The fluorescent microscope is the microscope which uses the fluorescence phenomenon to distinguish between things. For example, tuberculosis لسلا is an infectious disease caused by the bacterium Mycobacterium tuberculosis, in the diagnosis we take abiopsy from the lung, this biopsy contains human cells, matrix and bacterial cells, most cells are not fluorescent, but some bacteria like Pseudomonas Aeruginosa are fluorescent, to distinguish between the components of the biopsy, we can't use brightfield microscope because all of them will be stained, and we can't use darkfield microscope because all of them reflects light, so we must use a fluorescent dye which has a selective affinity for the bacteria (green for example) so everything appear black except the bacteria. Also, we can use more than one dye in this microscope, for example, nucleus of neurons are stained with a blue dye, the ER with a red dye and the skeleton with green dye. The usage of fluorescence microscope appears greatly in bacterial biofilms which are thousands of bacteria cells aggregated together (other name is extracellular polymeric matrix) and we used a treatment which could be antibiotic , disinfectant or any other antimicrobial treatment and we want to observe the exactly which cells are killed, so we use the fluorescent microscope with 2 different fluorescent dyes; one for viable cells(green) and one for died cells(red) so I can see the distribution of the killing among the bacteria in the tissue. It can also be used to see the distribution of the bacteria in a certain tissue and so on. One unique type of fluorescent microscope is the confocal microscope which scans many sections to produce one 3D image.

Structure: fluorescent microscope has a light source with an excitation filter which picks an exact color (excitation light) and reflect it to the sample from above (from the objective lens) because we need to see the emitted light only not the excitation light , if we have a green dye we need just the blue light from the light source and then a green light is emitted, there is also an emission filter (monochromatic filter) and we choose it depending on the dye's color, we need it because sometimes the excitation color is reflected from the sample so the filter guarantees that just the green light passes so we see an all-black background with a green cell, the images are very sharp and very clear.

A student's question : how do we make the affinity selective for one cell to another?

For example, peptidoglycans are found only in bacteria so we pick a dye that binds specifically to peptidoglycans. Also, human cells have an endoplasmic reticulum while bacterial cells do not, so we pick a dye that binds specifically to the ER.

But to distinguish between viable and dead bacterial cells we use 2 dyes ; green and red, the green dye molecules(SYTO 9 dye) are small, permeable and can pass through the functional and intact plasma membrane of the viable cells while red dye molecules can't pass because they are large but they can pass through the leaky non-functional plasma membrane of the dead cells because the integrity will be compromised, and also the green dye molecules will enter so the color of the emitted light is supposed to be umber(mix between red and green) but actually the emitted light from the dead cells will be perfectly red because red dye molecules quenches (absorbs) immediately the green light emitted from the green dye molecules but if the green dye is replaced by a blue dye the emitted light from dead cells will be mixed between red and blue colors.

B-Electron microscope: بعد العيد وكل عام وأنتم بخير

*Please refer to the slides for more images