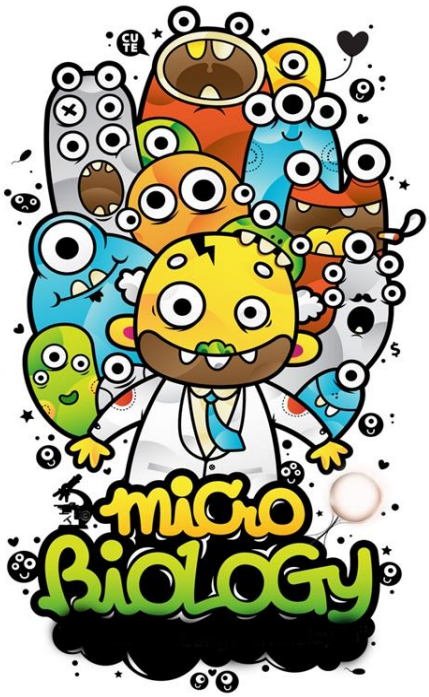

Subject: Microbiology- Electron Microscopy

Lecture Number: 2

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Microscopy 2

In the previous lecture we have talked about light microscope (bright-field, dark-field, phase contrast and fluorescent microscopes), and we have discussed how each one of them works. We said that you won't be able to distinguish any two objects as separated ones (resolution) if they are less than 0.2µm, but why?
The reason is: **diffraction limit**, which depends on the function of:

1- Refraction of index for the lenses.

2- Wavelength of the visualizing media.

The diffraction limit can be calculated by dividing lambda over 2 i.e. (λ/2). In other words, it equals to the 0.2 µm we have talked about. Accordingly, having any value under the diffraction limit: (λ/2), means that the resolution is lost.

Therefore, in order to be able to see and distinguish smaller particles we need to obtain lower diffraction limit and that could be theoretically obtained by decreasing the wavelength of light, however it is impossible to do so. This is due to the fact that wavelength depends on mass & velocity. The light`s mass is negligible and its velocity is fixed.

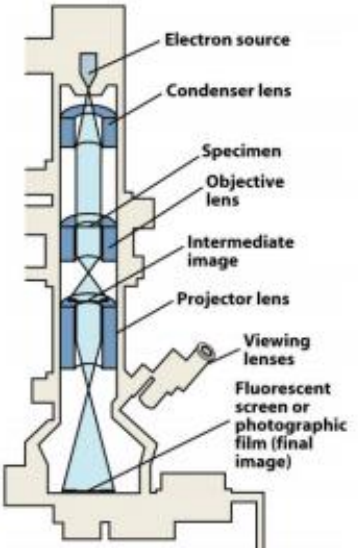
Accordingly, we resort to using **Electron Microscopy**.

Electrons are surrounded by negative charges that help the electron to accelerate when it is affected by an electrical field. Acceleration will lead to an increase in the velocity and therefore a decrease in the wavelength.
Ultimately, the diffraction limit is decreased.

E.g. If the wavelength decreased to 1 nm, diffraction limit would be 0.5 nm

Generally, the electron microscope utilizes a beam of electrons and electromagnetic lenses as oppose to light and glass lenses in the light microscope.

We have two different types of electron microscopes that we are concerned with:



1- Transmission Electron Microscope (TEM)

- **Naming**:
  It is called transmission because the image is viewed by the help of the electrons which are transmitted through the sample.
- **Structure and mechanism:**
  - In the TEM we have an electron source (electron gun) which provides us with beam of electrons.
  - This beam passes through the electromagnets then it is condensed and directed to pass through the sample.
  - The electrons can continue their path towards the sample easily due to the vacuum that is found above the sample which prevents electrons from collision with other air particles.
  - After that, the electron beam will be attenuated just like in the light microscope. Thus, the intensity of this beam will be changed depending on the object that is passing through, whether it results with high or low attenuation which leads to low and high intensity, respectively. So the difference in intensity plays a role in viewing the image.

Note: *In the light microscope, there will be a change in the color and intensity whereas in the electron microscope we only have a change in intensity, and that is because we can't see electrons at all, they have no color.*

- **The image:**
  In electron microscope we can't look at the image using only ocular lenses, for that reason we have what is called a **fluorescent screen** which transfers the difference in intensity of electrons into a difference in intensity of light. It translates the 2D images of areas with high intensity of electrons into an image with high intensity brightness, that's why we always have a non-colored image (dark white image)

Note (1): The colored images (from EM) that you see on internet are edited using Photoshop.
Note (2): Sometimes treatment with heavy metals (gold or platinum) is required to enhance the contrast.

- **Advantages** :
  Because we are using cross sections not the whole cell it helps us viewing small internal structures that other microscopes couldn't view.
- **Disadvantages**:
  1- 2D image, it depends on the intensity not the thickness; that's why we can't have a full image of the morphology of the sample.
  2- Limitation for the thickness of the slide: 100 nm, otherwise electrons will not be able to get through the sample. Slicing is achieved using a microtome.

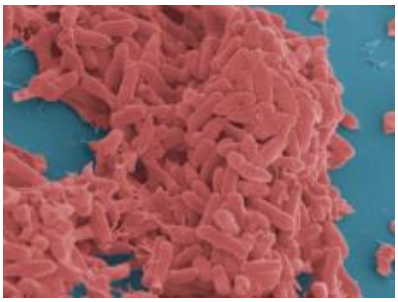
** 10 minutes have passed **

2- Scanning Electron Microscope (SEM)

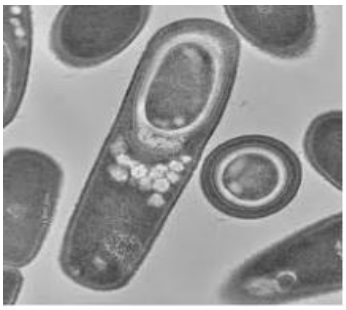
- **Naming**:
  It is used to view the outer surface of a cell and not concerned about the internal structures thus "Scanning".
- **Structure and mechanism:**
  -Like the TEM we have an electron gun gives beam of electrons, it is then condensed and then towards the sample
  - Unlike the electron microscope the SEM doesn't need the transmitted electrons, it uses the scattered electrons. The fluorescence screen (sensor) is found in a certain angle above the sample, it receives the scattered and emitted electrons from the sample, how?
  The falling angle of the electrons equals the reflected angle, so not all the reflected electrons would reach the sensor. Another way that electrons may reach the sensor is when the origin beam is absorbed by the sample and ionized then new emitted electrons may fall on the surface of that sensor. These two ways, depend on the topography of the sample.

Note (3): samples here, don't need to be thin, we don't use the microtome because we are looking for scattered electrons more than transmitted.

- Advantages: It displays the topography and architecture of the sample. It gives a 3D image that differs between thin and thick areas.
- Disadvantage: We can only see the surface of the sample. Less resolving power in comparison with TEM.



SEM image



TEM image

SEM

Light Microscopes

Sample Preparation:

Comprises two simple procedures:

1- Specimen preparation
2- Staining

1- Specimen preparation

There are two ways to prepare a specimen both of which start by putting the sample (e.g. bacteria) on the glass slide. A- Wet mount slide

B- Smear slide

A- For the Wet Mount Slide Technique:

- Collect a drop of the sample from a bacterial culture using a micro pipette. Then put it on the glass slide gently without removing the liquid around it, and that why it is called (wet mount).
- We use this technique when we are not using any stains because the staining procedures obligate us to put the dye solution on the sample and then to rinse it. If that happened, the drop of our sample will vanish, so it is not efficient if you are using satins.
- Therefore it is good to use while using the dark field and phase contrast microscope, in order to see the motility and the division of the cell.
- **Hanging Drop Slide** is a special type of wet mount, but is more efficient.

- When we use microscopes most of what is seen is the surface of the sample so it is hard to study the motility of the bacteria from different sites (some areas will not appear). Thus the hanging drop technique allows us to see the motility over an even surface.
- Hanging drop is underwent by placing a drop of microbial culture on a cover slip surrounded by petroleum jelly to prevent evaporation .The cover slip is then inverted over the well of a depression slide .
- There are some treatments that are agreed to be added to these procedures Like CMC (carboxymethyl cellulose) or some other hydrogens. These hydrogens are added when we need to study the division of a cell, it increases the viscosity of the cell in order to prevent it from moving and go out of site.

B- Smear slide

- Mainly used when we do staining, in this technique we use a tool called (inoculation loop) which is composed of a handle, tungsten wire, and at the end of the wire there's a ring which is used to get very small volumes of a liquid culture (less than 1 µm).
- The loop which is full of microbial culture is spread on a slide, allowed to air dry then passed quickly over a flame 3-4 times (heat fixation). **Heat fixation** causes the cells to adhere to the slide and alters them so they accept the stain.

If the sample is not dry before heat fixation, the microorganism will die due to the boiling of the liquid (e.g. water) - The microbial cells are usually killed (by heat) in this technique.

Inoculation loop

2- Staining

- We stain samples using dyes. A dye is a substance that has the ability to absorb light at certain wavelength, and bind to the wanted microorganism or bacteria.
- There are two major types of dyes :
  A- Anionic dyes (acidic dyes -ve)
  B- Cationic dyes (basic dyes +ve)

Note: most cells have a negative surface, so a cationic dye should be used since anionic dyes would cause repulsion. We use the anionic dye to stain the background of the cell.

30 minutes have passed

- The main purpose of using staining procedures in our experiments is to be able differentiate. E.g. differentiate between gram positive and gram negative in a procedure called gram staining.
- There are two major staining procedures:
  A- Simple staining: usually we use single cationic dye in order to enhance the contrast of the sample; it is just used to see the shape, size and morphological characteristics of the bacteria.
  B- Differential staining: (more commonly used) A staining procedure that helps us to differentiate between different types of microorganisms or different structures of the bacteria.
    - ❖ GRAM Staining:
      Is a type of differential staining that differentiates between the bacteria depending on the cell wall (gram positive and negative)
      → Structure of the cell wall of the gram positive bacteria : layer of peptidoglycan
      →Structure of the cell wall of the gram negative bacteria : much thinner layer of the peptidoglycan and a second membrane .

Note: special staining is used to visualize certain structure in the microorganism.

Note: gram non-reactive bacteria is neither positive nor negative bacteria, it is not stained by this procedure.

→ Gram staining procedure:

1- Get crystal violet solution and stain our sample by salting it into this solution for 30-60 seconds. This crystal violet will pass through the plasma membrane and enters the cell .

2. Iodine is then added as a mordant (i.e. a chemical that helps retain the stain in certain cells)

3. Ethanol (95%) or ethanol/acetone is used to decolorize the cells that cannot retain crystal violet; this is followed by rinsing with water

4. The cells are then counterstained with safranine (red/pink).

- The 4 groups of organisms that can be distinguished based on the Gram stain:

1. Gram positive: the cells retain crystal violet stain

2. Gram negative: the cells don't retain crystal violet but rather counterstain with safranine

3. Gram non-reactive: the cells don't (or poorly) stain

4. Gram variable: the cells stain unevenly

** تَجْرِي الرِّيَاحُ كَمَا تَجْرِي سَفِينَتُنَا..... نَحْنُ الرِّيَاحُ وَنَحْنُ الْبَحْرُ وَالسُّفُنُ **