STRUCTURE OF BACTERIAL CELLS.

MICROSCOPIC EXAMINATION OF BACTERIA

Minsk BSMU 2023

МИНИСТЕРСТВО ЗДРАВООХРАНЕНИЯ РЕСПУБЛИКИ БЕЛАРУСЬ
БЕЛОРУССКИЙ ГОСУДАРСТВЕННЫЙ МЕДИЦИНСКИЙ УНИВЕРСИТЕТ
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СТРУКТУРА БАКТЕРИАЛЬНОЙ КЛЕТКИ. БАКТЕРИОСКОПИЧЕСКИЙ МЕТОД ИССЛЕДОВАНИЯ

STRUCTURE OF BACTERIAL CELLS. MICROSCOPIC EXAMINATION OF BACTERIA

Учебно-методическое пособие

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INTRODUCTION

Methods of microbiological diagnostics are constantly being improved. This is primarily due to emergence newer diagnostic tools, techniques, and research instruments. Molecular approaches, highly sensitive and specific genetic and immunological methods of research reduce the time of diagnosis and increase the reliability of laboratory diagnostic tests. However, such a «classic» method as microscopy is still the most widely used, simple, cheap and rapid method for studying microorganisms. For some infectious diseases, microscopy is able to confirm the clinical diagnosis, for others it is a tool for preliminary diagnosis. The study of the morphology and structure of microorganisms is impossible without the use of microscopes.

The educational and methodical manual aims at providing detailed information about microscopic research method and its application for medical students. The text provides information on the morphology and structure of the bacterial cell, which we can obtain using microscopy. Special emphasis has been placed on microscope types, slide preparation techniques, and staining methods.

Minimum knowledge level expected for this topic learning:

– classification of life: The Three Domain Concept;
– major features of cellular organization of Prokaryotes and Eukaryotes;
– parts of compound microscope and their functions.

Control questions on the topic:

1. Basic bacterial cell structure: components of bacterial cell.
2. The composition and function of bacterial cell wall.
3. The cell wall of gram-positive and gram-negative bacteria.
4. Acid-fast bacteria and unique properties of their cell wall.
5. Microscopic method of examination: tasks, procedure, evaluation of the method.
6. Brightfield light microscope. The technique of oil immersion microscopy.
7. Preparation of the hanging-drop and wet-mount slides.
8. Darkfield, phase-contrast, fluorescence microscopy. Electron microscopy.
9. Fixed smear preparation and fixation. Simple methods of staining.
10. Differential method of staining. Gram stain: medical application, principles, procedure.

BACTERIAL CELL STRUCTURE

Bacteria are microscopic unicellular organisms and have a characteristic cellular organization of prokaryotes. The term «***prokaryotes***» means «primitive nucleus» (from Greek). The bacterial nuclear apparatus is neither enclosed in a

nuclear membrane nor associated with a nucleolus. Whereas **eukaryotes** have a defined true nucleus — a membrane-bound organelle where DNA is stored, and other membrane-bound organelles that perform specific functions in the cell.

The bacterial cell has several elements that allow it to function as a living organism. Basic structures of all bacteria are *genetic apparatus*, *ribosomes* and *cell membrane*. In addition, all bacteria (with the exception of mycoplasmas) have a stiff cell wall, located underneath the capsule (if there is one) and above the cell membrane (fig. 1).

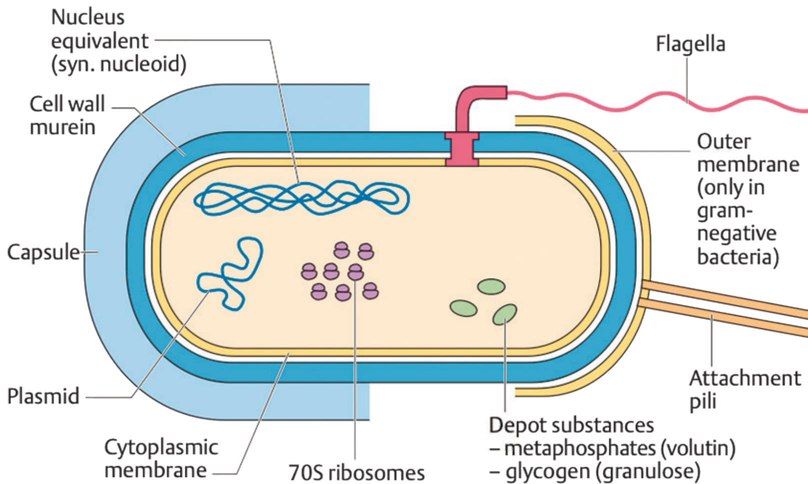

Fig. 1. Basic bacterial cell structure (Kayser F. H. [et al.], Medical Microbiology, 2005)

Cell membrane (plasma membrane, cytoplasmic membrane) is a thin (5–10 nm) delicate structure. It encloses the *protoplasm*, which consists of ***cytoplasm*** (an amorphous gel-like colloidal suspension composed of water, nutrients, metabolites, enzymes, and gases) with cell organelles and inclusion granules.

Cell membrane primarily contains phospholipids and proteins. The basic structure is a *phospholipid*, composed of a glycerol molecule attached a hydrophilic phosphate «head» and to two hydrophobic fatty acid «tails». Phospholipids are organized into two layers (*phospholipid bilayer*). A significant difference between the cytoplasmic membrane of prokaryotes and eukaryotes is the absence of sterols in the bacterial membrane (except in *Mycoplasma*). The embedded *proteins* act as facilitators in moving molecules through the membrane. Cytoplasmic membrane also contains enzymes associated with biosynthesis of polymers. Bacterial membrane usually has a higher proportion of protein than eukaryotic membranes.

Functions of cell membrane:

– it acts as a semipermeable selective barrier regulating the transport of materials into and out of cell;

– it participates in synthesis of cell wall components, DNA, and membrane lipids;

– it helps in electron transport and oxidative phosphorylation;

– it has the machinery for the synthesis and secretion of enzymes and bacterial toxins;

– it is the site of action of certain antibiotics, e. g. polymyxin.

Mesosomes. These are convoluted or multilaminate structures formed as invaginations (infoldings) of the cell membrane. Mesosomes increase the membrane surface area and are the principal sites of respiratory enzymes which are analogous to mitochondria in eucaryotes. The septal mesosome attached to the bacterial DNA is believed to coordinate DNA replication and cell division during binary fission.

Genetic apparatus. As mentioned above, prokaryotes do not have a discrete, well-defined nucleus. Instead, bacteria have a *nucleoid*, which is an irregularly-shaped region that contains the bacterial DNA. By analogy with eukaryotes, this can be termed a «chromosome». Many bacteria may also possess *extrachromosomal genetic elements* that give them additional beneficial properties.

Nucleoid. The essential genetic information of most bacteria is carried in a long, double-stranded, circular molecule of DNA with supercoiled structure and without histones. There are no introns in bacterial genome, instead, the DNA comprises a continuous coding sequence of genes. It is haploid (single copy), it is not surrounded by a nuclear membrane, it does not contain a mitotic apparatus and replicates by simple fission. To visualize the nucleoid, light microscopy after Feulgen staining, transmission electron microscopy and fluorescence microscopy are used.

Plasmids. These are small circular self-replicating molecules of extra-chromosomal double stranded DNA. Plasmids are 100–1000 times smaller than the nucleoid genome structure and carry a small number of non-essential genes. There may be only one or many plasmids in a bacterium. They are copied independently of the bacterial chromosome and can be transferred to other bacteria in a population, spreading genes that beneficial to survival.

Plasmid genes can encode resistance to antimicrobials (R-plasmids), some degradative functions (D-plasmids), and/or virulence (virulence-related adhesins, toxin production, hemolysins). Another two general types of plasmids are Col-plasmids (encode synthesis of bacteriocins (also known as colicins), which are proteins that kill other bacteria) and F-plasmids (contain transfer genes that allow genes to be transferred from one bacterium to another through conjugation — direct cell-to-cell contact by sex-pilus). So, plasmids are not required for bacteria metabolism and reproduction, but may confer certain functional benefits.

Ribosomes. The bacterial ribosomes are small cytoplasmic ribonucleoprotein particles whose primary function is to serve as the site of mRNA translation and protein synthesis. Although ribosomal function is the same in both prokaryotes and eukaryotes, organelle structure is different. Ribosomes are characterized as 80S and are comprised of 40S and 60S subunits in eukaryotes and 70S in prokaryotic cells (the letter «S» denotes the Svedberg unit, which measures the sedimentation coefficient of the ribosome in an ultracentrifuge). Bacterial 70S ribosome consists of two subunits — 30S (small subunit) and 50S (large subunit). Subunits of bacterial ribosome are specifically targeted by antimicrobials such as the aminoglycosides, macrolides, and tetracyclines. The difference in the structure of bacterial and eukaryotic ribosomes allows antibiotics to specifically inhibit protein synthesis in bacteria, but not in the eukaryotic cell. Bacterial ribosomes are never bound to other organelles, but are free-standing structures distributed throughout the cytoplasm.

Intracytoplasmic inclusions (or granules) are storage of nutrients/energy (polysaccharides, lipids, or polymerized metaphosphates) present in some bacteria. For example, polymetaphosphate or volutin or as metachromatic granules are found in *Corynebacterium species* and *Mycobacterium species*.

The **capsule (or glycocalyx)** is a structure located outside the cell membranes of certain bacteria. Most of the bacterial capsules are polysaccharide in nature, rarely contains proteins. *Bacillus anthracis* contains poly-D-glutamate capsule.

The capsule protects the bacterium from phagocytosis and facilitates adherence to surfaces. Capsule acts as an antigen and capsule`s polysaccharide often used for vaccine.

Cell wall. The bacterial cell wall is tough and rigid structure, surrounding the bacterium. The cell wall of bacteria is multilayered and weighs about 20–25 % of the dry weight of the cell.

The cell wall provides:

1. **Shape** of bacterial cells.
2. **Protection** (against osmotic pressure, environment and phagocytes).
3. **Permeability** (allows nutrients and small molecules such as water, mineral salts or simple metabolites to pass through).
4. **Communication** with the environment (contain receptors and other signaling molecules), involvement in binding to eukaryotic hosts' cell.
5. **Antigenic properties** of bacteria.

The most important structural element of the wall is a unique macromolecule murein (syn. peptidoglycan). Peptidoglycan is comprised of polysaccharide chains N-acetylglucosamine (GlcNAc, NAG) and N-acetylmuramic acid (MurNAc, NAM) that are crosslinked by peptides (fig. 2).

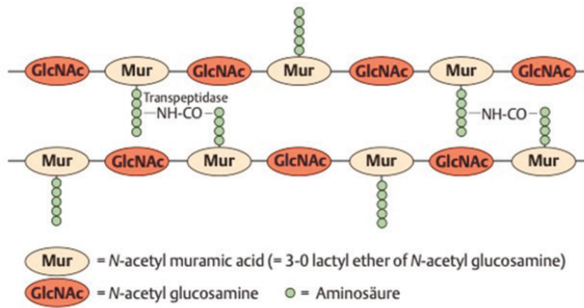

Fig. 2. The structure of murein (Kayser F. H. [et al.], Medical Microbiology, 2005)

Depends on the cell wall structure, all bacteria are divided into two groups, which differ in their pathogenicity and sensitivity to antibiotics. Differences in structure are clearly revealed by Gram staining of bacteria.

Gram-positive cell wall. The thick cell wall of Gram-positive bacteria consists mainly of peptidoglycan which often contains an interpeptide bridge (fig. 3).

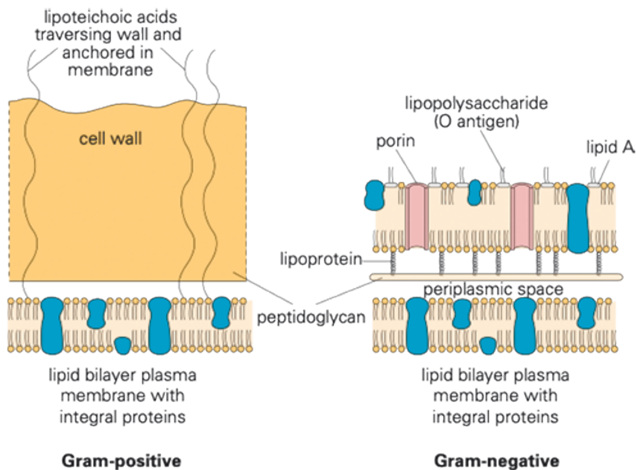

Fig. 3. The cell wall of Gram-positive and Gram-negative bacteria (Kayser F.H. [et al.], Medical Microbiology, 2005)

These cell walls also contain teichoic acids. Teichoic acids are connected either to the peptidoglycan itself or to the lipids of the plasma membrane: in this case, they are called lipoteichoic acids. Gram-negative bacteria lack of teichoic acids.

Gram-negative cell wall. The walls of Gram-negative bacteria are much more complicated than those of Gram-positive bacteria.

The Gram-negative cell wall outside the peptidoglycan layer (thin layer, about 10 % of the weight of the wall) contains three main components — lipoprotein layer, outer membrane, and lipopolysaccharides.

The *lipoprotein layer* is a small lipoprotein that is joined to the underlying peptidoglycan and embedded in the outer membrane by its hydrophobic end.

The *outer membrane* is a bilayer structure; its outer part contains a distinctive component called lipopolysaccharide (LPS), *outer membrane proteins* (Omps; OmpA with the murein lipoprotein form a bond between outer membrane and murein), *porins* (permit the passive diffusion of low-molecular-weight compounds), *outer membrane-associated proteins* (allow bacteria to attach to host cell receptors).

Lipopolysaccharides (LPS) are complex molecules containing both lipids and carbohydrates. Structurally, the LPS are made up of three parts:

1. *Lipid A* is a major constituent of the outer membrane. **Lipid A is the toxic portion (endotoxin)** and binds specifically to receptors (LPS receptor, or **TLR-4**) to activate macrophages. LPS can also non-specifically activate B cells without the help of T cells. LPS cause some of the symptoms that occur in Gram-negative bacterial infections.

2. *The central polysaccharide* is linked to lipid A. Its composition varies according to the species.

3. *The O side chain (O-antigen)* is a short polysaccharide chain extending beyond the central polysaccharide. It has a varies composition in different bacterial strains, according to antigen specificity and LPS can be serotyped to classify bacteria.

The cell wall of gram-negative bacteria contains *periplasmic space*. It is the space between the inner cell membrane and outer membrane. It encompasses the peptidoglycan layer.

Pili or fimbriae are hair-like structures on the outside of bacteria cells, it consists of helices of protein called *pilins*. There are pili of the 1st (general) type which are present in most bacteria and sex pili (2nd type): are used to exchange genetic material between cells during a process called ***conjugation***.

Pili are organs of adhesion and are responsible for colonization of bacteria.

Flagella are thread-like appendages, protruding from the cell wall, that confer motility to the bacteria (organs of locomotion). They made up of a protein flagellin. **Flagella** are highly antigenic, possess H-antigens, and some of the immune responses are directed against these proteins.

Some bacteria can have more than one flagellum. Depending on the number and location of bacterial flagella, they can be classified as *monotrichous* (bacteria have one polar flagellum), *lophotrichous* (several polar flagella), *peritrichous* (fla-

gella distributed over the entire surface of the bacterium), *amphitrichous* (flagella at both end), *atrichous* (do not possess any flagellum).

Acid fast bacteria have cell wall that is very rich in lipids and contains waxes, especially mycolic acid (branched-chain of hydroxy lipid), which prevents dye-binding (for example, *Mycobacterium tuberculosis*).

SHAPES OF BACTERIA

Bacteria have three basic shapes: spherical (round), rod shaped, or spiraled. A round bacterium is called a **coccus**. A rod-shaped organism is called a **bacillus** or simply a rod. A spiraled bacterium with at least two or three curves in its body is called a spirillum. Long organisms with many coils are called **spirochetes**.

Individual bacterial genera or species are often characterized by the cell patterns they form when grouping together as they multiply. Cocci may occur in pairs (*diplococci*), chains (*streptococci*), grapelike clusters (*staphylococci*), or packets of four (*tetrads*), and are rare found singly (fig. 4).

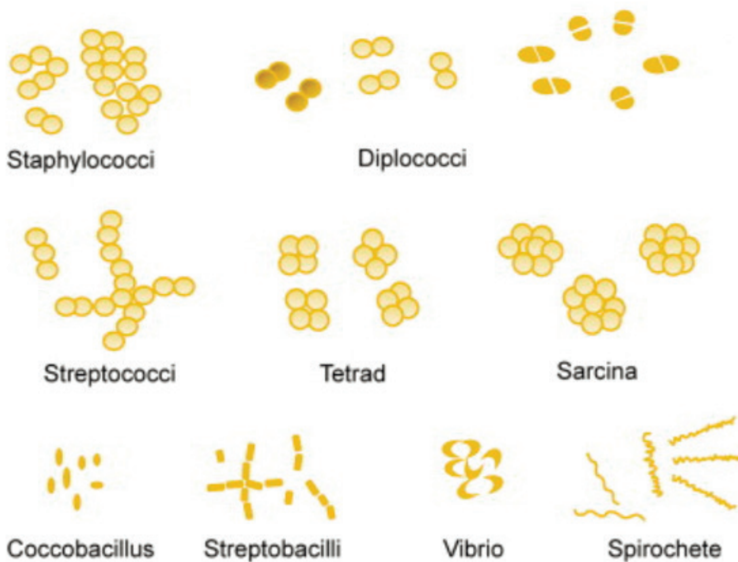

Fig. 4. Basic shape of bacteria (Source: https://www.sciencedirect.com)

Rod-shaped bacteria generally occur as individual cells, but they may appear end-to-end pairs (*diplobacilli*) or line up in chains (*streptobacilli*). Some species tend to palisade, that is, line up in bundles of parallel bacilli; others may form V, X, or Y figures as they divide. Some may show great variation in their size and length (known as *pleomorphism*). Spiraled bacteria occur singly and usually do not form group patterns.

BACTERIOSCOPIC METHOD

Bacterioscopic method is a set of techniques for detecting and studying of morphological and tinctorial properties of bacteria in the specimens by microscopy.

Specimens: laboratory culture, pathological samples, samples from the environment.

The main purpose — to establish the etiology of the disease, as well as determination of purity isolated pure culture.

The steps of the method:

1. Specimen collection (pus, sputum, blood, urine, feces, aspirates from the bronchi and stomach, the liquor, the nasal washes, autopsy, etc.).
2. Transporting the material to the laboratory in special containers.
3. Preparation of smear (if necessary, fixation and staining).
4. Microscopy of the slide.
5. Report.

Method evaluation:

Advantages: The process is simple, quick and cheap.

Disadvantages: low sensitivity (the required concentration of microbes is 10^4–10^5 in 1 ml); low specificity (due to the similarity in morphology of microorganisms of different species); risk of infection.

In laboratory practice, use of the following types of microscopic preparations:

1) **Native preparations** (***wet-mount; hanging drop***) are used to observe living bacteria. The poor contrast of such preparations makes it necessary to amplify this aspect (darkfield and phase-contrast microscopy). They are often used to determine the active motility of bacteria.

2) **Fixed (stained) preparations** (***bacteriological smear; thin smear; thick drop of blood***, etc.) are richer in contrast.

Native preparations technique

Wet-mount technique. A drop of the test material is placed into the center of a well-cleaned, fat-free slide and place a cover slip (free of air bubbles).

Hanging drop. The mobility and morphology of the bacteria are assessed in the hanging drop.

Technique: To prepare this kind of preparation, *special glass slides with an impression (well) in the center are utilized*.

1. The deepening of a hollow-section slide is framed with vaseline (adhesive).
2. A drop of the test material (young liquid culture) is placed on a cover slip using a sterile loop. The slide coated with vaseline is now pressed onto the cover slip in such a way that the cover glass sticks to the vaseline without the drop touching the slide.
3. The slide is now quickly turned over so that the drop hangs freely inside the hollow section on the cover glass.

Fixed preparation technique

Bacteria are difficult to observe directly with a bright-field microscope because they are very small and colorless, so it is necessary to prepare bacterial samples in a special way.

A bacterial smear — a small amount of culture spread in a very thin film on the surface of the slide.

Smear require only a small amount of bacterial culture. If too much microbial culture is used, a large number of bacterial cells will concentrate on the slide. This reduces the amount of light that can pass through the specimen and makes it difficult to visualize the morphology of individual bacteria.

A good smear is one that appears as a *thin whitish layer of film when dry.*

Smears from broth cultures and cultures from solid media require different preparation techniques.

a) **Broth cultures**:

Using a sterile inoculating loop, apply one to two drops of broth culture to the center of the slide and gently spread the liquid on the surface of the slide (about 1 cm in diameter). Allow the smear to air dry completely.

b) **Cultures from solid medium**:

These cultures must be diluted: place a drop or two of 0.9 % NaCl solution in the center of a glass slide; then take some of the culture with a sterile bacterial loop and spread it in a drop of saline. The finished smear should cover an area of about 1 cm and be a translucent whitish film. Allow the smear to dry completely.

Fixation is necessary to attach microorganisms to the glass slide and preserve their structure in its original state.

Heat fixation is performed by the rapid passage on the air-dried smear two or three times over the flame of the burner (fig. 5). During heat fixation, the bacterial proteins are coagulated and fixed to the glass surface.

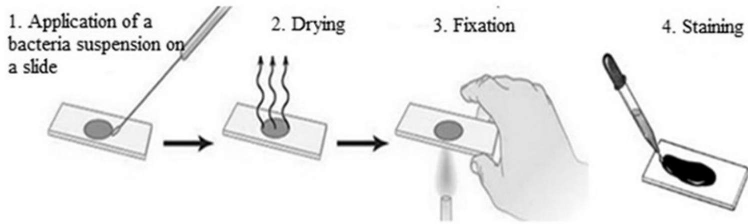

Fig. 5. Heat-fixed smear preparation

Chemical fixation used to protect cell morphology as well as cell fine substructures of the more delicate microorganisms. Chemical fixative gets inside the cells and cross-links components present in cells, such as most proteins and nucleic acid. Fixatives are acids and aldehydes such as acetic acid, glutaraldehyde, etc.

Type of microscopy

Microscopy is the technical field of using microscopes to view objects that cannot be seen with the unaided eye.

There are currently two fundamental types of the microscopy: ***light microscopy*** and ***electron microscopy***. Light microscopy uses glass lenses to focus visible light, while electron microscopy uses electromagnetic lenses to focus a beam of electrons.

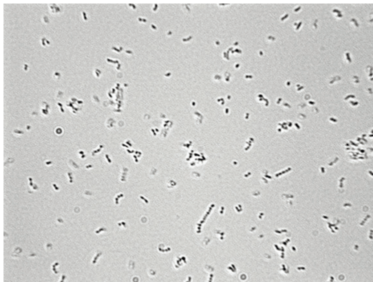

Fig. 6. Organisms viewed through a bright field microscope stand out as dark objects against a light background

Brightfield Microscopy uses **transmitted visible light** to develop magnified images. Visible light can be natural (sun) or artificial (source of illuminations).

In this type of microscopy, the microscope field is bright, while microorganisms are dark because they absorb part of the light (fig. 6).

1) ***Magnification***. In light microscopy, light passes through a condenser, then through a sample, and then through a series of magnifying lenses.

Condenser lenses do not affect magnification. They are positioned between the light source and the sample and are required to focus the light onto the sample.

There are two lens systems for enlarging objects in the microscope: the ocular lens is located in the eyepiece; the objective lens — in the nosepiece. These

lenses magnify an object 1000x when viewed through a 10x ocular lens and a 100x objective lens.

2) ***Resolution*** is the minimum distance between two points for them to be perceived as separate points rather than a fused image. High resolution means very fine details are visible.

The resolution limit of the best light microscope is 0.2 micrometers. This is sufficient to observe the general morphology of a prokaryotic cell, but not enough to discriminate between virus-sized particles. Therefore, to visualize very small objects, an electron microscope is used (the resolution limit is up to 1 nanometer).

It is very important to know that *in order to achieve maximum resolution in light microscopy, high power objectives (100x lens) and special immersion oil must be used*.

Using an immersion oil can improve your resolution, but why?

There is a small amount of air between the sample and the lens. When light passes from glass to air, it is diffracted due to the difference in refractive index between two materials; the air diffuses the rays of light before they can be focused by the lens. To solve this problem, *a drop of oil must be used to fill the space between the sample and an oil immersion lens*, a special lens designed for use with immersion oils. The oil has almost the same refractive index as glass, and light rays pass through it with little refraction.

3) ***Contrast***. Bacteria are essentially transparent and practically do not contrast with the background of the sample. One way to solve this problem is to stain the bacteria with one of a number of dyes. The types and properties of dyes will be discussed shortly.

Darkfield Microscopy uses **reflected light** to create a magnified image.

This is similar to the ordinary light microscope; however, the condenser system is modified (by adding a special disk called *a stop*) so that the specimen is not directly illuminated. The condenser *directs the light obliquely* so that the field of the microscope is dark and the microorganisms are bright because the light is deflected by them (fig. 7).

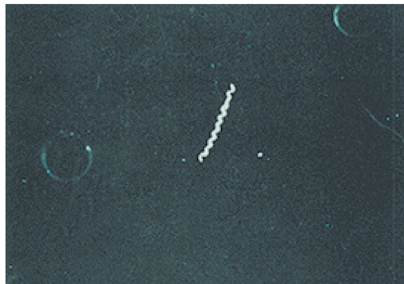

Fig. 7. Organisms viewed through a dark-field microscope stand out as bright objects against a dark background

Darkfield microscopy is used to observe live unstained specimens and bacterial motility. For example, dark field microscopy can detect members of the order *Spirochaetales*. These thin, spiral-shaped microorganisms stain poorly and are difficult to see with brightfield microscopy.

Phase-Contrast Microscopy. The internal components of a living, un-

stained cell is difficult to distinguish because the contrast is insufficient. But cellular structures have different densities and alter the transmitted light in different ways. The phase-contrast microscope uses exactly this difference between the refractive indices of the cells and the surrounding.

Special microscope optics convert the difference between transmitted and refracted light, which leads to a significant variation in light intensity (*phase shift*) and thus to an increase in image contrast. The image appears dark against a light background (the denser material appears darker).

Phase-contrast microscopy is used to observe living cells and cellular structures.

Differential Interference Contrast Microscopy is exploited **the differences in refractive indices** as light passes through different materials.

This microscope has a device for splitting light into two beams that pass through the specimen and then recombine. The light waves are out of phase and when they recombine the three-dimensional (3-D) image is obtained. This microscope is used in unstained and transparent samples to improve their image contrast (fig. 8).

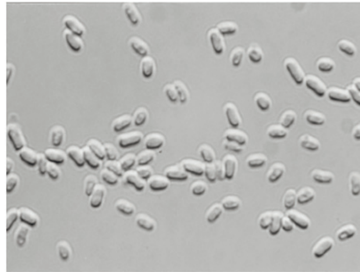

Fig. 8. The 3-D image under Differential Interference Contrast Microscopy

Fluorescence Microscopy is a microscopy technique that uses **the phenomenon of luminescence**. Some compounds called fluorochromes can absorb short-wavelength ultraviolet light and emit energy at a higher visible wavelength. Although some microorganisms show natural fluorescence, fluorescent microscopy typically involves staining organisms with *fluorescent dyes.*

The fluorescent microscope uses a high-pressure mercury, halogen, or xenon vapor lamp that emits a shorter wavelength of light than that emitted by brightfield microscopes. A series of filters are used to block the heat generated from the lamp, eliminate infrared light, and select the appropriate wavelength for exciting the fluorochrome. The light emitted from the fluorochrome is then magnified through traditional objective and ocular lenses. Organisms and specimens stained with flu-

orochromes appear brightly illuminated against a dark background, although the colors vary depending on the fluorochrome selected.

The **Confocal Scanning Laser Microscopy** — is a microscopy technique that uses **a focused laser beam**. The laser microscope is equipped with *an additional «confocal» device* — a pinhole diaphragm that blocks out-of-focus light during imaging.

The advantage of this type of microscopy is the acquisition of high-resolution 3D images of biological samples. Confocal laser microscopy allows studying the structure of cells, the dynamics and development of cells and tissues. If certain substances in biological samples are first stained with a fluorescent dye, the exact location of these compounds in the cell can be determined.

Electron microscopy is a microscopy technique that uses **a beam of electrons** to create a highly magnified image of microscopic specimens.

In an electron microscope, the specimen is illuminated by **a beam of electrons**, and the focusing is done **by electromagnets** instead of optics.

The components of the microscope must be completely evacuated so that the molecules in the air do not interfere with the movement of the electrons. Therefore, the electron microscope is an expensive, bulky unit of equipment. In addition, complex specimen preparation is required, which excludes the examination of living cells.

In the case of **transmission electron microscopes**, the electron beam must pass through the sample unhindered, so the samples must be carefully and thinly prepared, fixed and dehydrated. As the electrons pass through the specimen, images are formed by directing the electrons onto the photographic film, revealing the internal cell structures.

Scanning electron microscopes only evaluate the surface properties of objects, as a narrow beam of electrons scans back and forth as it reflects off the sample's surface. This creates a three-dimensional image of superficial rather than intracellular structures.

X-ray crystallography is not a typical microscopy technique and **uses soft X-rays** to obtain a magnified image of a specimen. However, it is currently the most powerful way to see the atomic structure of tiny molecules. When an X-ray beam passes through a crystal, diffraction patterns are generated which are then analyzed by special computer software to calculate the structure of the molecules. In this way, scientists have revealed the structure and function of many biological molecules, including vitamins, drugs, proteins, and nucleic acids such as DNA. Protein structures are usually represented in 3D models as colored threads and ribbons.

Modern microscopy uses a variety of optical instruments to perform complex examinations, but the traditional brightfield microscope is «*the workhorse*» and is used in all biological laboratories.

Bacteria staining

The size of the bacterial cells is about 0.2–2 µm in diameter, the length can vary between 1 and 10 µm. Stains are required to visualize bacteria and demonstrate the detail of their structures.

Tinctorial properties of bacteria — the ability of bacterial cells to perceive (bind) various dyes (this is determined by the structure and composition of the cell wall).

All cells as well as dyes exhibit some types of charge.

Acidic Dyes — are **Anionic**. These type dyes have an affinity for the Positive components of a cell (attracted to *basic cell components* (cytoplasmic proteins)).

Example — India Ink, Acidic Fuchsine, Eosin.

Acidic dyes are sometimes used to stain backgrounds against which colorless cells can be seen, a technique called ***negative staining***.

Basic Dyes — are **Cationic**. These type dyes have an affinity for the Negative components of a cell (attracted *to acidic cell components* (nucleic acids, ribosomes)).

Example — Methylene Blue, Crystal Violet, Basic Fuchsine, Safranin, Malachite Green.

A high concentration of DNA and ribosomal RNA in a bacterial cell makes it more sensitive to basic dyes. In this regard, almost exclusively basic dyes are used in microbiological practice.

If both nuclear and cytoplasmic structures are to be stained in a given preparation, combinations of acidic and basic dyes may be used. A common example is the hematoxylin (basic) and eosin (acidic), stain used in the examination of tissue sections (H & E).

Simple stains

A simple stain is using an aqueous or alcohol solution of **a single dye**.

Some of the common simple stains in the lab are methylene blue (aqueous), crystal violet (an alcohol solution) and basic or carbol fuchsin.

Simple Stain Technique:

1. Apply dye to the fixed smear for the required time.
2. Rinse with tap water.
3. Place the smear in an upright position, allow excess water to drip off and allow the smear to dry. Alternatively, you can blot dry.
4. Examine the stained smear under a 100x (oil) immersion microscope objective.

Methylene Blue stains may be performed on cerebrospinal fluid. This may be useful to detect the *H. influenzae* and *N. meningitidis*.

Carbol fuchsin stains is used to show the morphology of delicate bacteria (carbolic fuchsin achieves a swelling effect).

Differential stain

Differential staining requires the use of **several dyes**, which are applied sequentially to a heat-fixed smear.

The first dye is called **the primary stain**. Its function is to impart its color to all cells.

The second reagent is called **the decolorizing agent**. Based on the chemical composition of cellular components, the decolorizing agent may or may not remove the primary stain from the cell.

The final dye, **the counterstain**, has a color that contrasts with the primary stain. It can only color a cell if the decolorizing agent has removed the primary dye from the cell.

Thus, cell types or cell structures can be differentiated from each other on the basis of retained staining.

Gram stain

The most important differential stain used in bacteriology is the Gram stain. The Gram stain allows the bacteria to be divided into Gram-negative and Gram-positive with regard **to the structure of their cell wall**.

The principle of Gram stain is presented in the table 1.

Table 1

Principle of Gram stain

Chemical reagents	Principle
Primary Stain: **Crystal Violet**	This violet dye is used first and stains all cells purple
Mordant: **Iodine**	This reagent serves as a mordant, a substance that increases the cells affinity for a stain. It forms an insoluble complex crystal violet/iodine (CV-I) serves to enhance the color of the stain. At this point, all cells will appear purple-black
Decolorizing agent: **Ethanol, 95 %**	In Gram-negative cells, alcohol increases the porosity of the cell wall by dissolving lipids in the outer layers. The CV-I complex is washed out of the thin peptidoglycan layer, leaving the cells colorless (uncolored). Gram-positive cells have a much thicker peptidoglycan layer and few lipids, so they strictly retain the CV-I complex. In addition, the pores are reduced due to the dehydrating effect of alcohol. Thus, the firmly bound primary dye complex is difficult to remove and the cells remain purple
Counterstain: **Basic fuchsine**	This is final reagent, used to stain pink those cells that have been previously decolorized. Gram-positive bacteria retain the **purple color** of the primary stain. Gram-negative bacteria appear **pink**

Gram's stain can also be used to identify fungi and some protozoa. These various applications demonstrate the versatility of Gram's stain.

Gram's Stain Technique:

1. Place the heat-fixed smear on a staining rack and overlay the surface with crystal violet solution. After 1 minute of exposure to the crystal violet stain, wash thoroughly with tap water.
2. Overlay the smear with Lugol's solution (iodine solution) for 1 minute.
3. Hold the smear between the thumb and forefinger and flood the surface with a few drops of the alcohol, until no purple color is washed off. This usually takes 30–40 seconds.
4. Wash under running water and place the smear back on the staining rack. Overlay the surface with fuchsine counterstain for 1 minute.
5. Rinse with running water.
6. Place the smear upright in a staining rack to allow excess water to drain and allow the smear to dry. Alternatively, one may blot dry.
7. Examine the stained smear under the 100× (oil) immersion objective of the microscope.

Gram-positive bacteria stain blue-violet, Gram-negative bacteria stain pink.

Acid fast stain

Acid Fast Staining used to differentiate acid-fast & non-acid fast bacteria.

The Ziehl-Neelsen is the oldest method used but requires heating the specimen during the staining procedure. Many laboratories have replaced this method with either the cold acid-fast stain (Kinyoun method) or the fluorochrome stain (auramine-rhodamine method).

Ziehl-Neelsen stain. Fixed smears are first stained by a **strong carbol fuchsin with the application of heat**. Heating facilitates entry of phenolic carbol fuchsin stain into the bacteria. It is then decolorized with 5–20 % (depending on the bacteria to be stained) sulfuric acid. It is then counterstained with a contrasting dye, such as **methylene blue**. The acid-fast bacilli retain the red color of carbol fuchsin and appear bright red in stained smears; others take on the color of the counterstain - blue.

Endospore stain

Members of certain Gram-positive genera including *Bacillus* and *Clostridium* form a special type of dormant cell, an endospore, that is resistant to destruction. The spore wall is relatively impermeable, but dyes can be made to penetrate it by heating the preparation.

Special staining methods proposed by Ozheshko, Peshkov, and others are used.

Ozheshko's stain. A smear is dried in the air, treated with 0.5 per cent sulphuric acid, and heated until it steams.

Then, the preparation is washed with water, dried, fixed above the flame, and stained by the Ziehl-Neelsen's technique. Spores stain pink-red, the cell appears blue.

Special stains to observe cell structures

Dyes can also be used to stain specific structures inside or outside the cell.

Capsules do not stain well; negative staining is used to detect them.

Negative staining (with Indian ink). The ink preparation is a negative representation of the bacteria.

Technique:

A drop of ink is placed on the end of a completely grease-free slide, and a small drop of the material to be examined is placed next to it. The two drops are mixed and smeared thinly like a blood smear. Allow the preparation to air dry.

Capsules are visualized as a colorless halo around microbial bodies against a dark background (fig. 9).

Loeffler and Neisser staining is used to detect the *volutin granules*.

The Neisser method is used to identify or prove *Corynebacterium diphtheriae*. Cells are stained with methylene blue and crystal violet in acidic solution and then counterstained with chrysoidin solution. The polar bodies (volutin granules) are therefore black-blue in a light yellow-brown colored bacterial body (fig. 10).

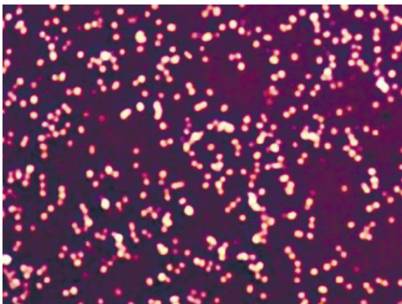

Fig. 9. Since the capsule substance does not absorb the dye, it appears as a colorless gap in the ink film that surrounds the cell body

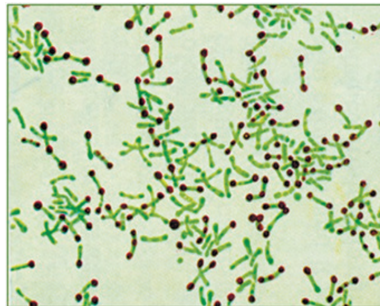

Fig. 10. The volutin granules which appear reddish-pink when stained with methylene blue (Neisser stain)

The Loeffler method involves staining the preparation with Loeffler's solution.

Loeffler's solution: 30 mL of methylene blue in alcohol solution + 100 mL 0.01 % KOH solution in water.

Some microscopical method used in the microbiology laboratory are summarized in the table 2.

Table 2

Some microscopical method used in the microbiology laboratory

Method	Principle and application
	Direct Examination
Wet mount Handing drop	Unstained preparation is examined by brightfield, darkfield, or phase-contrast microscopy
India ink	*Negative staining* with India Ink is used to detect *capsules*
	Differential stains
Gram stain	Most commonly used stain in microbiology laboratory, forming basis for separating major groups of bacteria (e. g., gram-positive, gram-negative)
Silver impregnation method	Cells and structures that *are too thin* to be visualized by the light microscope can be rendered visible by impregnation of silver on their surface. Silver impregnation method is a common method used for *staining spirochetes and some small bacilli*
Romanowski-Giemsa stain	Used to detect of many bacteria (e. g., spiral-shaped borreliae, treponemas, or leptospira) as well as for protozoans, and mammalian cells (e. g., for blood cell count). Giemsa stain combines methylene blue and eosin. Eosin ions are negatively charged and stain basic components of cells orange to pink, whereas other dyes stain acidic cell structures various shades of blue to purple
Ziehl-Neelsen stain	**Acid-fast stain**. Used to differentiate acid-fast & non-acid fast bacteria
Ozheshko stain	**Endospore stain**. Used to detect the *endospore*
	Special stains
Loeffler and Neisser stain	Used to detect the *volutin granules*
	Fluorescent stains
Acridine orange stain	Used for detection of bacteria and fungi in clinical specimens. Dye intercalates *into nucleic acid* (native and denatured). At neutral pH, bacteria, fungi, and cellular material stain reddish-orange. At acid pH (4.0), bacteria and fungi remain reddish-orange, but background material stains greenish-yellow
Auramine-rhodamine stain	Same principle as a*cid-fast stains*, except that fluorescent dyes (auramine and rhodamine) are used for primary stain, and potassium permanganate (strong oxidizing agent) is the counterstain and inactivates unbound fluorochrome dyes. Organisms fluoresce yellowish-green against a black background
Direct fluorescent antibody stain	Used for detecting or identifying many organisms (e. g., *Streptococcus pyogenes*, *Bordetella*, *Francisella*, *Legionella*, *Chlamydia*, *Pneumocystis*, *Cryptosporidium*, *Giardia*, *influenza virus*, *herpes simplex virus*). Antibodies are complexed with fluorescent molecules. Specific binding to an organism is detected by presence of microbial fluorescence

TASKS FOR INDEPENDENT WORK

Learning test

1. What do all eukaryotes have that prokaryotes do not?

a) Nucleus;
b) Flagella;
c) DNA;
d) Cell membrane.

2. Bacterial capsule can be best demonstrated by:

a) Gram staining
b) Acid-fast staining
c) Negative staining
d) Loeffler staining

3. Which structure's role is to prevent white blood cells engulfing the bacteria?

a) Capsule;
b) Pilus;
c) Flagella;
d) Peptidoglycan;
e) Fimbriae.

4. What is the size of ribosomes in procaryotes:

a) 30S;
b) 70S;
c) 50S;
d) 80S.

5. Cell wall of gram-positive bacteria:

a) contains lipopolysaccharide;
b) consist of 1–2 layers of peptidoglycan;
c) consist of up to 40 layers of peptidoglycan;
d) contains teichoic and lipoteichoic acids;
e) contains an outer membrane.

6. Cell wall of gram-negative bacteria:

a) contains lipopolysaccharide;
b) consist of 1–2 layers of peptidoglycan;
c) consist of up to 40 layers of peptidoglycan;
d) contains teichoic and lipoteichoic acids;
e) contains an outer membrane.

7. What does the capsule look like after negative staining?

a) Black zone around bacteria;
b) Red zone around bacteria;
c) Colorless halo around bacteria.

8. Chemical agent used in Gram stain:

a) Basic fuchsine;
b) Cristal violet;
c) Ethanol;
d) Indian ink;
e) Iodine.

9. Lipopolysaccharide is a component of cell wall of:

a) Gram-positive bacteria;
b) Gram-negative bacteria;
c) Virus;
d) Fungi.

10. What is the aim of phase-contrast and darkfield microscopy?

a) microscopy of preparation stained with fluorescent dyes;

b) microscopy of native (not stained) preparation;

c) microscopy of fixed and stained preparation.

Questions for self-control:

1. What is the most crucial step in performing the Gram stain procedure? Explain your answer.
2. How can you increase the resolution on your microscope?
3. What types of microscope preparations are there? What is the difference?
4. Why are basic dyes more successful in staining bacteria than acidic dyes?
5. What part of the bacterial cell is most involved with Gram staining?
6. Are acid-fast bacteria gram positive or gram negative? Explain your answer.
7. Why are endospores so difficult to stain?
8. *Due to the holidays, your regular classes were canceled and Gram staining was performed on long-incubated cultures. When examining stained preparations of staphylococci, pink cocci were found, located in clusters. Consider the result.

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CONTENTS

Introduction..3

Bacterial cell structure 3

Shapes of bacteria 9

Bacterioscopic method..10
  Native preparations technique 10
  Fixed preparation technique...11
  Type of microscopy 12
  Bacteria staining 16
  Simple stains...16
  Differential stain 17
  Special stains to observe cell structures...19

Tasks for independent work 21

Literature...22

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