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SCHOOL OF BIO AND CHEMICAL ENGINEERING

DEPARTMENT OF BIOTECHNOLOGY

B.SC- MICROBIOLOGY

UNIT I- GENERAL MICROBIOLOGY - SMB1101

The History and Scope of Microbiology

I. What is microbiology?

A. Microbiology is the study of organisms and agents that are generally too small to be seen clearly by the unaided eye. These organisms include viruses, bacteria, algae, fungi, and protozoa.

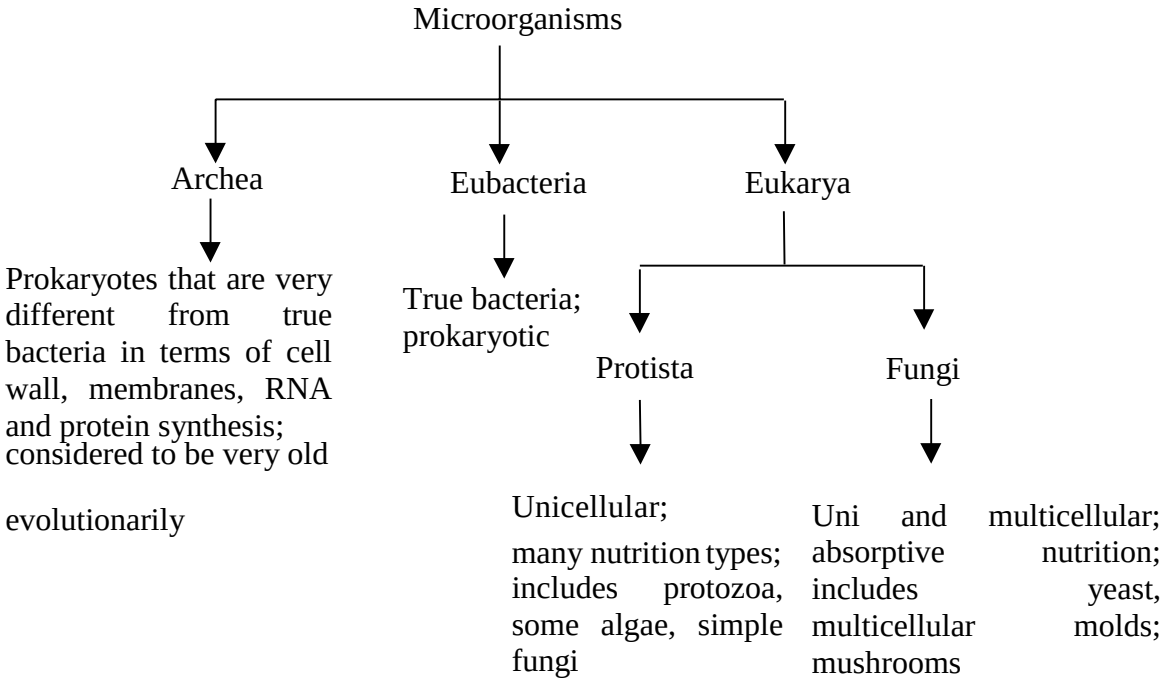
B. Microbiology can be applied or basic.

C. Microbiology is linked to many other scientific disciplines including biochemistry, cell biology, evolution, ecology.

D. Subdisciplines (both applied and basic research)

1. General microbiology: broad range of microbiological questions
2. Medical microbiology: microbes that cause human disease
3. Public health and epidemiology: Studies and controls transmission, frequency, and distribution of disease
4. Immunology: the immune system
5. Agricultural microbiology: impact of microbes on agriculture
6. Microbial ecology: relationships between microbes and their habitats
7. Food microbiology: Prevention of food borne disease; microbes that make food and drink
8. Industrial microbiology: commercial use of microbes to produce products
9. Biotechnology: manipulation of organisms to form useful products

II. What are microbes?



III. The scope and relevance of microbes
   A. L. Pasteur "The role of the infinitely small in nature is infinitely large."
   B. 1st living organisms
   C. Live in every possible environment
   D. More numerous than macroorganisms
   E. Constitute the largest component of biomass
   F. Fundamental to the ecosystem
   G. Have changed the course of history in obvious and not so obvious ways

IV. History
   A. Discovering the "organisms"
      1. 1676: A. Leeuwenhoek – first to observe and describe microbes accurately
      2. 1884: C. Chamberland – constructed a bacterial filter that allowed the identification of viruses
      3. 1898: Loeffler and Frosch – identified filterable infectious agent as cause of foot-and-mouth disease in cattle
      4. 1898-1900: M. Beijerinck – identified tobacco mosaic virus
      5. 1982: S. Prusiner – described prions (infectious protein that causes a particular normal protein to alter its shape and become a prion)
   B. Disproving spontaneous generation (that living organisms could develop from nonliving matter)
      1. 1688: F. Redi – first to challenge theory of spontaneous generation by showing that if raw meat was protected from flies, the formation of maggots was prevented
      2. 1748: R. Needham – supported spontaneous generation of microbes by showing that even after boiling mutton broth and pouring into sealed containers, growth of microbes occurred
      3. 1776: L. Spallanzani - challenged spontaneous generation as it pertained to microbes by showing that sealed containers that were boiled do not produce microbes
      4. 1861: L. Pasteur – rigorously disproved spontaneous generation
         a) filtered air □ showed that air contained microbial organisms
         b) constructed flasks with curved neck that allowed air into the flasks while dust, etc. remained in the neck □ placed broth into the flasks and boiled □ showed that no microbial growth resulted unless flasks were tipped to allow the broth into the neck
   C. The germ theory of disease
      1. Previously, people thought that disease was punishment for an individual's crimes, due to poisonous vapors, and/or an imbalance of the "four humors".
      2. First proponents of the idea that invisible organisms caused disease were Lucretius (B.C.) and Fracastoro (1546)
      3. 1835: A. Bassi showed that silkworm disease was due to a fungus.

4. 1867: J. Lister showed that antiseptic surgical procedures reduced the frequency of wound infections.
5. 1876/1884: R. Koch definitively proved that *Bacillus anthracis* caused the disease anthrax in cows and *Mycobacterium tuberculosis* caused the disease tuberculosis using Koch's postulates.
    a) The suspected pathogen should be present in ALL cases of the disease and NOT present in healthy animals.
    b) The suspected pathogen should be grown *in vitro* in pure culture.
    c) Cells from a pure culture of the putative pathogen should cause disease in healthy animals.
    d) The putative pathogen should be reisolated from the infected animal.

D. Preventing disease by vaccination
1. E. Jenner inoculated people with cowpox to protect against smallpox.
2. 1885 – Pasteur developed the rabies vaccine.
3. 1890: von Behring and Kitasato produced antibodies to purified toxins to protect against diphtheria and tetanus.
4. 1884: E. Metchnikoff described phagocytosis of bacteria.

E. Discovering the effect of microbes on organic and inorganic matter
1. 1856: Pasteur described lactic acid fermentation; contributions to wine industry.
2. 1887-1900: S. Winogradsky and M. Beijerinck studied soil microbes and their role in the biochemical cycles of sulfur, carbon, nitrogen

V. Recent history of microbiology – the 20 th century

A. Infectious diseases: The etiological agent of most infectious diseases has been ascertained. Current research focuses on understanding the molecular mechanisms by which disease is caused.

B. Chemotherapy: Discovery of antibiotics; antibiotic resistance

C. Immunology develops as a science.

D. Physiology and biochemistry: Using microbes as a model, many physiological and biochemical processes have been elucidated.

E. Genetics: Many of the advances in molecular genetics were made using bacteria as models. A few of the many:
1. 1941: Beadle and Tatum – 1 gene = 1 enzyme
2. 1943: Luria and Delbruck – mutations are spontaneous in nature
3. 1944: Avery, MacLeod, and McCarty – DNA is the genetic material
4. 1961: Jacob and Monod – the operon and gene regulation

F. Molecular biology: Many of the advances in molecular biology were made using bacteria as models. A few of the many:
1. 1970: Restriction enzymes discovered
2. 1979: Insulin synthesized using recombinant techniques
3. 1990: Gene therapy trials begin
4. 1995: The nucleotide sequence of the first free-living organism (*Haemophilus influenzea*) published

The Theory of Spontaneous Generation

The Greek philosopher Aristotle (384–322 BC) was one of the earliest recorded scholars to articulate the theory of **spontaneous generation**, the notion that life can arise from nonliving matter. **Aristotle** proposed that life arose from nonliving material if the material contained *pneuma* ("vital heat"). As evidence, he noted several instances of the appearance of animals from environments previously devoid of such animals, such as the seemingly sudden appearance of fish in a new puddle of water.[1]

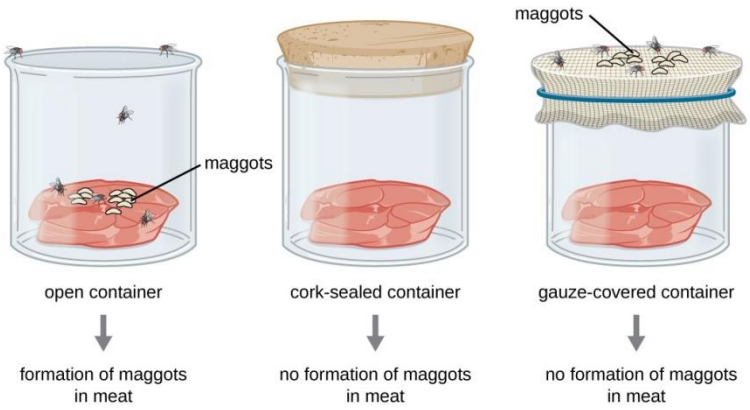
This theory persisted into the seventeenth century, when scientists undertook additional experimentation to support or disprove it. By this time, the proponents of the theory cited how frogs simply seem to appear along the muddy banks of the Nile River in Egypt during the annual flooding. Others observed that mice simply appeared among grain stored in barns with thatched roofs. When the roof leaked and the grain molded, mice appeared. Jan Baptista **van Helmont**, a seventeenth century Flemish scientist, proposed that mice could arise from rags and wheat kernels left in an open container for 3 weeks. In reality, such habitats provided ideal food sources and shelter for mouse populations to flourish.

However, one of van Helmont's contemporaries, Italian physician Francesco **Redi** (1626–1697), performed an experiment in 1668 that was one of the first to refute the idea that maggots (the larvae of flies) spontaneously generate on meat left out in the open air. He predicted that preventing flies from having direct contact with the meat would also prevent the appearance of maggots. Redi left meat in each of six containers (Figure 1). Two were open to the air, two were covered with gauze, and two were tightly sealed. His hypothesis was supported when maggots developed in the uncovered jars, but no maggots appeared in either the gauze-covered or the tightly sealed jars. He concluded that maggots could only form when flies were allowed to lay eggs in the meat, and that the maggots were the offspring of flies, not the product of spontaneous generation.

In 1745, John Needham (1713–1781) published a report of his own experiments, in which he briefly boiled broth infused with plant or animal matter, hoping to kill all preexisting microbes. He then sealed the flasks. After a few days, Needham observed that the broth had become cloudy and a single drop contained numerous microscopic creatures. He argued that the new microbes must have arisen spontaneously. In reality, however, he likely did not boil the broth enough to kill all preexisting microbes.

Lazzaro Spallanzani (1729–1799) did not agree with Needham's conclusions, however, and performed hundreds of carefully executed experiments using heated broth. As in Needham's

experiment, broth in sealed jars and unsealed jars was infused with plant and animal matter. Spallanzani's results contradicted the findings of Needham: Heated but sealed flasks remained clear, without any signs of spontaneous growth, unless the flasks were subsequently opened to the air. This suggested that microbes were introduced into these flasks from the air. In response to Spallanzani's findings, Needham argued that life originates from a "life force" that was destroyed during Spallanzani's extended boiling. Any subsequent sealing of the flasks then prevented new life force from entering and causing spontaneous generation (Figure 2).



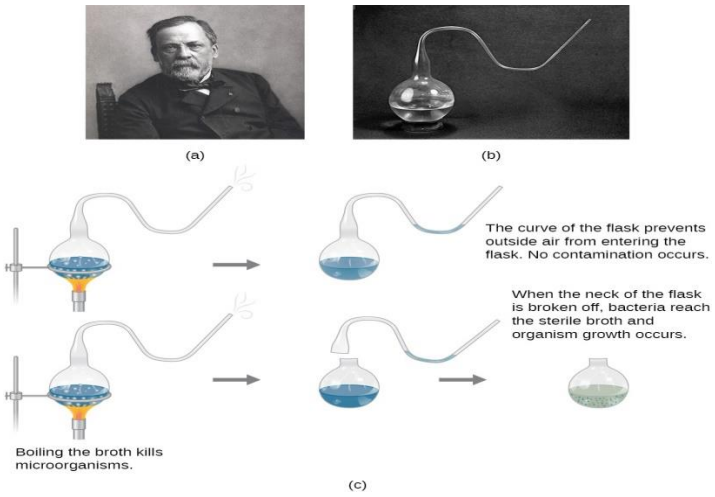
Disproving Spontaneous Generation

The debate over spontaneous generation continued well into the nineteenth century, with scientists serving as proponents of both sides. To settle the debate, the Paris Academy of Sciences offered a prize for resolution of the problem. Louis Pasteur, a prominent French chemist who had been studying microbial fermentation and the causes of wine spoilage, accepted the challenge. In 1858, Pasteur filtered air through a gun-cotton filter and, upon microscopic examination of the cotton, found it full of microorganisms, suggesting that the exposure of a broth to air was not introducing a "life force" to the broth but rather airborne microorganisms.

Later, Pasteur made a series of flasks with long, twisted necks ("swan-neck" flasks), in which he boiled broth to sterilize it (Figure 3). His design allowed air inside the flasks to be exchanged with air from the outside, but prevented the introduction of any airborne microorganisms, which would get caught in the twists and bends of the flasks' necks. If a life force besides the airborne microorganisms were responsible for microbial growth within the sterilized flasks, it would have access to the broth, whereas the microorganisms would not. He correctly predicted that sterilized broth in his swan-neck flasks would remain sterile as long as the swan necks remained intact. However, should the necks be broken, microorganisms would be introduced, contaminating the flasks and allowing microbial growth within the broth.

Pasteur's set of experiments irrefutably disproved the theory of spontaneous generation and earned him the prestigious Alhumbert Prize from the Paris Academy of Sciences in 1862. In a

subsequent lecture in 1864, Pasteur articulated "*Omne vivum ex vivo*" ("Life only comes from life"). In this lecture, Pasteur recounted his famous swan-neck flask experiment, stating that "life is a germ and a germ is life. Never will the doctrine of spontaneous generation recover from the mortal blow of this simple experiment." To Pasteur's credit, it never has.



Biogenesis Theory

The theory of biogenesis states that living things can only arise from living things and cannot be spontaneously generated. Learn more about this popular microbiology theory to better understand what it means.

The Spontaneous generation hypothesis proposed by scientists to explain the origin of the "animalcules" observed by Antoni van Leeuwenhoek in his magnifying lenses had received wide acceptance all over Europe from Antoni's time until the time of Louis Pasteur. Erroneous experimental set up, results, and conclusions of some scientists had supported and strengthened the hypothesis.

For example, the Englishman John Needham claimed that vital life is needed for the spontaneous generation of microbes. He added that the reason why no living organisms emerged from heated and sealed solutions in containers is that the "vital life" was destroyed by the heat and new "vital life" was not supplied to the solutions because they cannot enter the sealed containers.

Fortunately, there were scientists skeptical about the hypothesis, so they designed their own experimental set up and from the results they gathered, they drew the most feasible explanation on the origin of the "animalcules".Among the scientists was the Italian Lazzaro Spallanzani who opposed Needham's idea of the "vital life".

Proponents and opponents of spontaneous generation hypothesis debated a lot starting from the time Leeuwenhoek presented his discoveries (1670s) to the public until the time of Rudolf Virchow, who in 1858 challenged the spontaneous generation with his concept and definition of biogenesis.

This concept claims that living cells can arise only from preexisting living cells. Virchow defended this concept to the scientific community but he did not come up with a convincing

experiment to back up his idea. In 1861, the French scientist Louis Pasteur resolved the issue on the origin of microbes (“animalcules") through a series of ingenious and persuasive experiments.

Pasteur showed that microorganisms exist in the air and can contaminate sterile solutions, but he emphasized that air itself does not produce microbes. He filled a number of short-necked flasks with beef broth and then boiled their contents. He immediately sealed the mouths of some of the flasks while he left the others open and allowed to cool.

After few days, the contents of the unsealed flasks were found to be contaminated with microorganisms. No evidences of growing microorganisms were found on the sealed flasks. Pasteur concluded that the microorganisms in the air were responsible for contaminating non-living matter like the broths in John Needham’s flask.

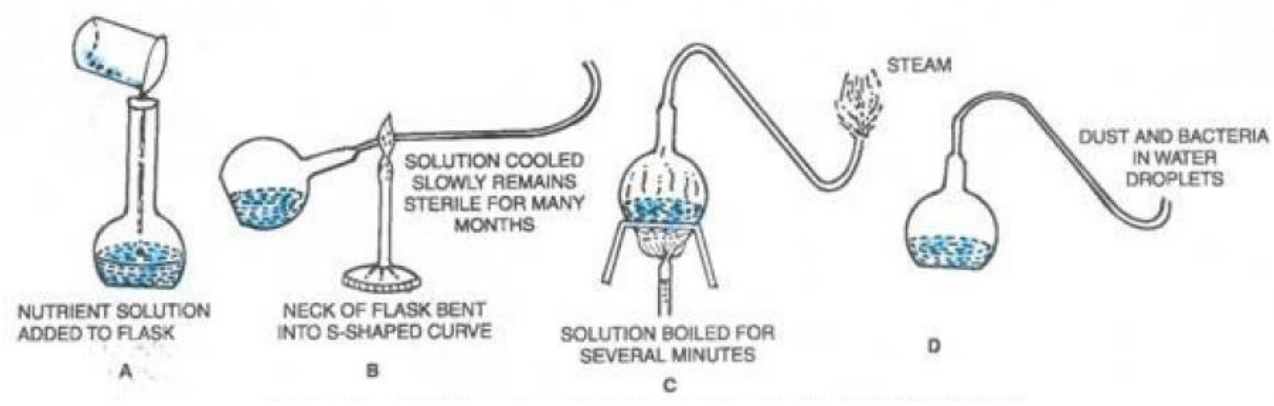
Pasteur performed another experiment but this time he put beef broth in open-ended long-necked flasks. He bent the necks of the flasks into S-shaped curves and boiled the contents of the flasks. Amazingly, the contents of the flasks were not contaminated even after several months.

The unique S-shaped design of Pasteur’s flasks allowed air to pass but trap microorganisms that may contaminate the broths. Do you know that some of the original vessels used by Pasteur in his experiments are still displayed in the Pasteur Institute, Paris today? A few of the flasks contain broths that remain uncontaminated for more than 100 years!

Pasteur demonstrated the presence of microbes in non-living materials whether they are solid, liquid, or air. In addition, he laid the foundation of aseptic techniques, techniques that prevent contamination by unwanted microbes.

These techniques are based on Pasteur’s idea that microbes can be killed by heat and that procedures can be designed to inhibit the access of airborne microbes to nutrient environment. Application of aseptic techniques is now the standard practice in medical and laboratory procedures.

Disproving the idea that microorganisms spontaneously generated from non-living matter through mystical forces is one of the greatest contributions of Pasteur in science. He provided the evidence that any appearance of “spontaneous" life in nonliving solutions can be attributed to microbes that already exist in the air or in the fluids themselves.



Contributions Of Antony Van Leeuwenhoek & Louis Pasteur

1. He first discovered & reported bacteria(1676).
2. Observed Microscopic structure of seeds & embryos of plants & some invertebrates.
3. He discovered Spermatozoa & RBCs.
4. He discovered characteristic microbes of human mouth, curd, vinegar.
5. Emphasized the abundance of these microorganisms..

Contributions Of Louis Pasteur

1.Disproved the theory of Spontaneous Generation.
2. Developed vaccines against Rabies, Anthrax, & Cholera.
3.Devised the purposes of destroying bacteria's by Pasteurization .
4. Proposed Germ Theory Of Disease.
5. Helped to improve the fermentation process during hi period in France.
6. Known As Father Of Microbiology.

LOUIS PASTEUR (1822-1895)

Louis Pasteur was born on December 27, 1822 in Dole, in the region of Jura, France and was trained as a chemist.

1. He disproved the widely accepted myth of spontaneous generation. He demonstrated that both fermentation and putrefaction were initiated by air borne microbes.
2. He described the scientific basis for fermentation, winemaking, and the brewing of beer.
3. His discovery that most infectious diseases are caused by germs, known as the "germ theory of disease", is one of the most important in medical history.
4. He discovered the method for the attenuation of virulent microorganisms that is the basis of vaccination. He developed vaccines against rabies, chicken cholera, anthrax, swine erysipelas and silkworm diseases.
5. He introduced the methods of sterilization, namely the steam sterilizer, autoclave and the hot air oven.
6. He proved the importance of cotton wool as stoppers for protecting media in flasks or tubes.
7. He had realized the importance of the constituents of the nutrients contained in a culture media, the pH and the oxygen.
8. Pasteur developed "pasteurization", a technique by which harmful microbes in perishable food products are destroyed using heat, without destroying the food.
9. He discovered the existence of life without oxygen. That is, discovered that bacteria can survive in anaerobic conditions.
10. Coined the term "vaccine" to commemorate the first successful vaccination against smallpox by Jenner.

Pasteur Institute, a private, state-approved institute financed by international public funds was inaugurated on 1888.

Robert Koch:

Robert Koch was a German doctor who did a lot of work in the field of microbiology. He was very successful in his work and was awarded the Nobel Prize in 1905 for his work and research on the study of diseases.Robert Koch made many contributions to the field of microbiology. He was able to develop specific ways to attack bacteria. He also was able to determine what bacteria were the cause of anthrax. Robert Koch developed a way to make it easier to observe bacteria. This was the known as the Petri dish. He also found the causes to various diseases such as tuberculosis, cholera, and typhus. Finally, Robert Koch made it easier to see bacteria under a microscope by staining it.

Robert Koch's work made significant advances in the treatment of diseases.

He was the first person to discover the precise cause of anthrax to be the bacteria *Bacillus anthracis* through the use of cultures. It is this discovery that paved way for a breakthrough in the area of infectious diseases. In the 1880's, Koch developed a guideline known as Koch's four postulates, to be used in establishing the cause of infectious diseases and which is the standard procedure used in laboratories to date. By following Koch's postulates, one is able to arrive at the causative agent of a disease through isolation. In other words, one is able to identify the exact microorganism that causes a certain disease. He was also interested in the cholera epidemic that hit various countries in the world and even though another scientist had already isolated the causative agent of cholera, Koch's identification of the bacterium Vibrio cholera was more conclusive. Robert Koch was also the first individual to dispel the assertion that tuberculosis is an inherited ailment by distinguishing its causative agent to be *Mycobacterium tuberculosis* in 1882. Koch mentored many students who through the use of his four postulates discovered the disease-causing agents of syphilis, pneumonia, tetanus and leprosy among others. His extensive works in the field of microbiology earned him several accolades and he is even referred to as the father of microbiology.

Robert Koch was actually named Heinrich Herman Robert Koch, and is widely considered the founder of the field of Microbiology. His most important achievements include his studies relating to tuberculosis and to the transmission of anthrax between cows and humans. Koch helped move forward our understanding of blood-born diseases as well as was in which you

could grow cultures of bacteria in order to try various methods of eradication and treatment. He discovered that tuberculosis, which killed 1 in 7 people in the mid 19th century, was caused by the bacteria Tubercle Bacilli. This would be the first crucial step in the treatment of the disease. He won the Noble Prize for this achievement.

However, he also postulated many theories that have since been proven regarding the nature of disease. He said that in order to establish that an organism causes a diseases, it must adhere to certain principals, such as it must be absent in healthy organisms, it can be cultured in a lab and it can produce an original infection. These postulates are the basis for Microbiology.

Edward Jenner:

FRS FRCPE (17 May 1749 – 26 January 1823) was an English physician and scientist who pioneered the concept of vaccines including creating the smallpox vaccine, the world's first vaccine. The terms *vaccine* and *vaccination* are derived from *Variolae vaccinae* (smallpox of the cow), the term devised by Jenner to denote cowpox. He used it in 1798 in the long title of his *Inquiry into the Variolae vaccinae known as the Cow Pox,* in which he described the protective effect of cowpox against smallpox.

Jenner is often called "the father of immunology", and his work is said to have "saved more lives than the work of any other human". In Jenner's time, smallpox killed around 10% of the population, with the number as high as 20% in towns and cities where infection spread more easily. In 1821, he was appointed physician extraordinary to King George IV, and was also made mayor of Berkeley and justice of the peace. A member of the Royal Society, in the field of zoology he was the first person to describe the brood parasitism of the cuckoo. In 2002, Jenner was named in the BBC's list of the 100 Greatest Britons.

Paul Ehrlich (1854-1915) in 1904 found that the dye Trypan Red was active against the trypanosome that causes African sleeping sickness and could be used therapeutically. This dye with antimicrobial activity was referred to as a 'magic bullet'. Subsequently in 1910, Ehrlich in collaboration with Sakahiro Hata, a japanese physician, introduced the drug Salvarsan (arsenobenzol) as a treatment for syphilis caused by Treponema pallidum. Ehrlich's work had laid important foundations for many of the developments to come and the use of Salvarsen

marked the beginning of the eni of chemotherapy and the use of chemicals that selectively inhibit or kill pathogens without causing damage to the patient.

Edward Jenner (1749-1823) an English physician was the first to prevent small pox. He was impressed by the observation that countryside milk maid who contacted cowpox (Cowpox is a milder disease caused by a virus closely related to small pox) while milking were subsequently immune to small pox. On May 14^{th} , 1796 he proved that inoculating people with pus from cowpox lesions provided protection against small pox. Jenner in 1798, published his results on 23 successful vaccinators. Eventually this process was known as vaccination, based on the latin word 'Vacca' meaning cow. Thus the use of cow pox virus to protect small pox disease in humans became popular replacing the risky technique of immunizing with actual small pox material.

Jenner's experimental significance was realized by Pasteur who next applied this principle to the prevention of anthrax and it worked. He called the attenuated cultures vaccines (Vacca = cow) and the process as vaccination. Encouraged by the successful prevention of anthrax by vaccination, Pasteur marched ahead towards the service of humanity by making a vaccine for hydrophobia or rabies (a disease transmitted to people by bites of dogs and other animals). As with Jenner's vaccination for small pox, principle of the preventive treatment of rabies also worked fully which laid the foundation of modern immunization programme against many dreaded diseases like diphtheria, tetanus, pertussis, polio and measles etc.

The Germ Theory of Disease

- Before the Germ theory of disease, the causes suggested for the occurrence of disease were the effect of supernatural phenomena like planetary alignments, effect of bad bodily humors and the faulty environment.
- For example, in ancient Greece, it was thought that disease was spread not via direct contact with other infected individuals, but rather via infectious "seeds" in the air or food products.
- The predominant theory until germ theory of disease was eventually accepted in the 19th century was termed "miasma theory", meaning "pollution" or "bad air".
- Miasma theory stipulated that disease originated from the decomposition of organic matter, causing a noxious vapor harboring disease-causing agents. It was believed that individuals

could contract disease by inhaling foul-smelling air associated with contaminated drinking water, unsanitary conditions, and air pollution.

- The germ theory is applied to infection control in hospitals, the treatment of food and water, and efforts to control the spread of infection in natural settings. Example: The various vaccination and disease prevention programs.
- Even in the present day, research continues to identify the microbes responsible for diseases, to rapidly and accurately detect their presence, and to devise strategies that will minimize or completely prevent the particular diseases.
- The practices of disinfection, sterilization, personal hygiene, and proper food preparation have their basis in germ theory.
- Knowledge that microorganisms can cause disease spawned efforts to prevent the microbes from coming into contact with people, food, water, and other materials.
- Knowledge that many diseases are caused by microorganisms, and that the microbes can be spread from person-to-person and from an inanimate surface to a person spurred the development of techniques to minimize or prevent microbial spread. One example is asepsis—the treatment of living and non-living surfaces to kill or prevent the growth of associated microorganisms.

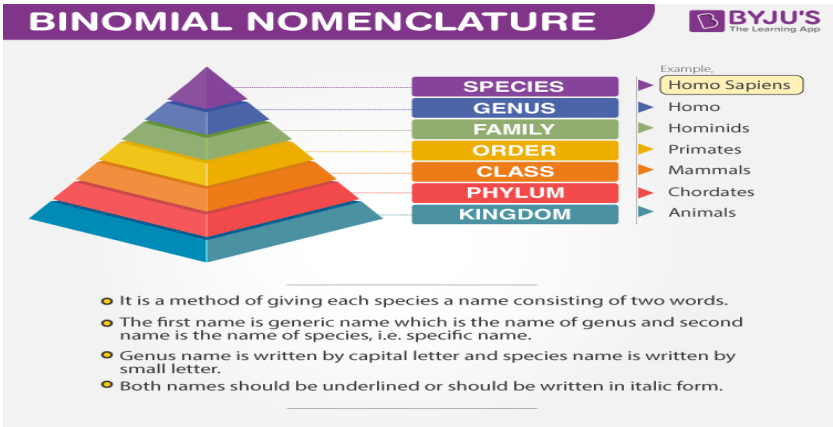
- However, with the subsequent development of new experimental work, research and new theories of different researchers surfacing, the older theories were replaced. Nonetheless, the new theory has withstood scientific scrutiny for centuries.
- The germ theory of disease is the currently accepted scientific theory of disease.

Binomial Nomenclature:

The system of binomial nomenclature was introduced by Carl Linnaeus. Multiple local names make it extremely difficult to identify an organism globally and keep a track of the number of species. Thus, it creates a lot of confusion. To get rid of this confusion, a standard protocol came up. According to it, each and every organism would have one scientific name which would be used by everyone to identify an organism. This process of standardized naming is called as Binomial Nomenclature.

All living species including plants, animals, birds and also some microbes have their own scientific names. For eg.,

- The scientific name of the tiger is presented as *Panthera tigris*. '*Panthera'* represents the genus and '*Tigris'* represents a particular species or specific epithet.
- The scientific name of humans is presented as *Homo sapiens*. '*Homo'* represents the genus and '*sapiens'* represents a particular species.
- The Indian bullfrog is scientifically written as *Rana tigrina. 'Rana'* is the name of the genus and '*tigrina'* is the name of the specific species.



Rules of Binomial Nomenclature

A Biologist from all over the world follows a uniform set of principles for naming the organisms. There are two international codes which are agreed upon by all the biologists over the entire world for the naming protocol. They are:

- International Code of Botanical Nomenclature (ICBN) – Deals with the biological nomenclature for plants.
- International Code of Zoological Nomenclature (ICZN) – Deals with the biological nomenclature of animals.

These codes make sure that each organism gets a specific name and that name is globally identified.

The naming follows certain conventions. Each scientific name has two parts:

- Generic name
- Specific epithet

The rest of the **binomial nomenclature rules** for writing the scientific names of organisms include the following:

1. All the scientific names of organisms are usually Latin. Hence, they are written in italics.
2. There exist two parts of a name. The first word identifies the genus and the second word identifies the species.
3. When the names are handwritten, they are underlined or italicized if typed. This is done to specify its Latin origin.
4. The name of the genus starts with a capital letter and the name of the species starts with a small letter.

Why is Binomial Nomenclature Important?

As stated previously, there are millions of species of organisms distributed throughout the world. Furthermore, the same organisms are known by different names around the world and this can cause confusion when trying to identify or classify. Hence, binomial nomenclature was seen as a viable solution to this problem.

Drawbacks of Binomial Nomenclature

Some of the basic drawbacks of binomial nomenclature are:

- If two or more names are currently in use, according to the law of priority, the correct name will be the one used first and the others end up being synonyms as validity is the senior synonym. Providing stability in the naming and classification of organisms must be emphasized.
- Also, the names used prior to those included in the "Systema Naturae", by Linnaeus are not recognized.

Five Kingdom Classification

Scientists have been trying to classify living organisms in various ways for centuries. In fact, even Aristotle classified living organisms on the basis whether they lived on land, water or air. But biologists wanted a broader system of classifying living organisms.

Five Kingdom Classification

Very early on, scientists began grouping the living organisms under different categories. Some biologists classified organisms into plants and animals. Ernst Haeckel, Robert Whittaker, and Carl Woese are some biologists who attempted a broader system of classification. Amongst these, the Five Kingdom Classification proposed by Robert Whittaker stood out and is widely used.

Whitaker proposed that organisms should be broadly divided into kingdoms, based on certain characters like the structure of the cell, mode of nutrition, the source of nutrition, interrelationship,

body organization, and reproduction. According to this system, there are five main kingdoms. They are:

- Kingdom Monera
- Kingdom Protista
- Kingdom Fungi
- Kingdom Animalia
- Kingdom Plantae

Kingdoms are divided into subgroups at various levels. The following flowchart shows the hierarchy of classification.

Kingdom → Phylum → Class → Order → Family → Genus → Species

Diversity in Living Organisms - Part 1

Classification of Organisms

Arranging organism into groups based on similarities and differences.

Advantages

- Identify organisms
- Finds interrelationship between organisms
- Makes studying organisms easy

Types of Classification

Cell Nature

- **Prokaryotic** — Nucleus and cell organelles are not demarcated
- **Eukaryotic** — Clear nucleus and membrane-bound cell organelles

Cell Occurrence

- **Unicellular** — Made up of single cell
- **Multicellular** — Multiple cells in clusters to form specialized tissue and organs

Mode of Nutrition

- **Autotrophs** — Make their own food
- **Heterotrophs** — Depend on others for food

Level of Body Organization

Five Kingdom Classification

*Given by Robert Whittaker

Kingdom Monera	Kingdom Protista	Kingdom Fungi	Kingdom Plantae	Kingdom Animalia
•Prokaryotic •Unicellular •Autotrophs/ Heterotrophs •Some have cell-wall, some doesn't	•Eukaryotic •Unicellular •Autotrophs/ Heterotrophs •Movement – Cilia, Flagella, Pseudopodia	•Eukaryotic •Mostly multicellular •Heterotrophs - Saprophytic •Cell wall – Chitin •Some form symbiotic association with blue-green algae	•Eukaryotic •Multicellular •Autotrophs •Cell wall - Cellulose	•Eukaryotic •Multicellular •Heterotrophs •Cell wall - absent
Bacteria	Amoeba	Mushroom	Plant	Lion

Binomial Nomenclature

*Introduced by Carl Linnaeus

Genus name + Species name → Scientific name

Rules to follow while naming organism through binomial nomenclature :

1. Names are written in *italicized* form.
2. First letter of genus should be capital.
3. First letter of species should be in lowercase.

Homo sapiens

4. If scientific texts are handwritten, they should be underlined.

Examples:

Panthera tigris

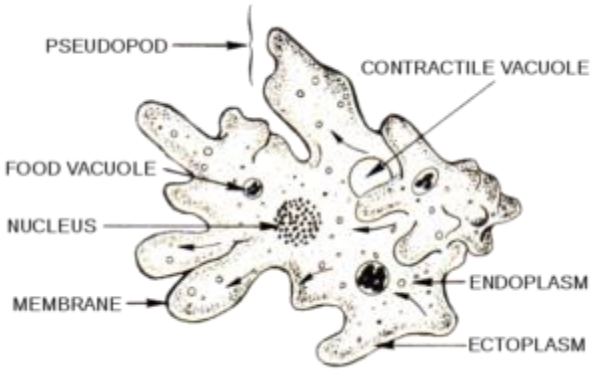
Mangifera indica

Kingdom Monera

These organisms are prokaryotic and unicellular. They do not have a well-defined nucleus and also lack cell organelles. Some organisms show the presence of cell wall while there are others without a cell wall. Consequently, some organisms are autotrophic and others are heterotrophic. Examples include *Bacteria, Cyanobacteria, and Mycoplasma.*

Kingdom Protista

Organisms grouped under Kingdom Protista are all unicellular, but eukaryotic organisms. These are the simplest forms of eukaryotes that exhibit either autotrophic or heterotrophic mode of nutrition. Some organisms have appendages such as cilia or flagella or pseudopodia to move around. Some examples are *Diatoms, Protozoans like Amoeba, Paramoecium*



Kingdom Fungi

Heterotrophic, Multicellular and Eukaryotic organisms are grouped under Kingdom Fungi. Their mode of nutrition is saprophytic as they use decaying organic matter as food. They have cell walls, which are made up of a substance called Chitin. Fungi also form a symbiotic association with some blue-green algae. *Yeast, Mushroom, Aspergillus* are examples of Fungi.

Kingdom Plantae

These are Eukaryotic, Multicellular organisms with a cell wall that is made up of cellulose. They are autotrophs and synthesize their own food through the process of photosynthesis. This kingdom includes all plants.

Based on the body differentiation and presence or absence of specialized vascular tissue, Kingdom Plantae is divided into different divisions, namely Thallophyta, Bryophyta, Pteridophyta, Gymnosperms, and Angiosperms. Examples are *Spirogyra, Ferns, Pines, and Mango Plant* etc.

Kingdom Animalia

This Kingdom includes organisms that are Multicellular, Eukaryotic, without the presence of cell wall. They have a heterotrophic mode of nutrition. They also exhibit great diversity. Some organisms are simple while others have a complex body with specialized tissue differentiation and body organs.

The Animal Kingdom is divided into many phyla and classes. Some of the phyla are Porifera, Coelenterata, Arthropoda, Echinodermata, Chordata etc. Examples – *Hydra, Starfish, Earthworms, Monkeys, Birds* etc.

It was realized by the biologists during recent past (1970s-80s) that the classification based on genetic characteristics (rather than phenotypic ones) with respect to evolutionary relatedness (phylogeny) of organisms may prove more stable and predictable.

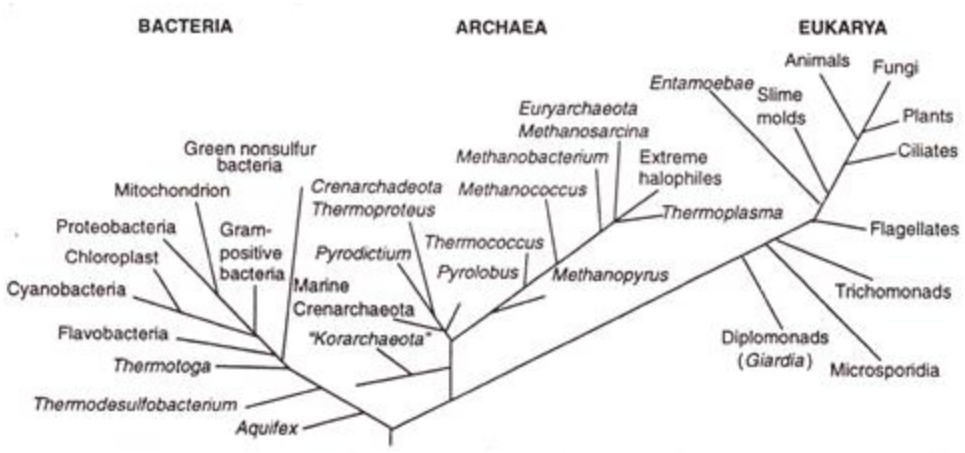
In 1987, Carl Woese of University of Illinois (USA) summarized ten years of work and proposed a phylogenetic classification system for prokaryotic species based on the nucleotide sequence of 16S rRNA molecules, the RNA of small subunit of prokaryotic ribosome.

He concluded that 16S rRNA molecule sequences could not only be used for comparative analysis between different species of prokaryotes but also between prokaryotic and eukaryotic species to provide a tree of relatedness based on common ancestry or genealogy because both prokaryotic and eukaryotic cells contain small subunit rRNA (SSU rRNA).

Ribosomal RNA (rRNA) is considered to act as a good genetic indicator of evolution of one organism from the other because it is functionally constant, universally distributed, and moderately well conserved in sequence across broad phylogenetic distances.

Also, because the number of different possible sequences of ribosomal RNA is so large that the similarity in two sequences always indicates some phylogenetic relationship. However, it is the degree of similarity in ribosomal RNA sequences between two organisms that indicates their relative evolutionary relatedness.

The studies of Carl Woese and his colleagues based on comparative sequencing of 16S (prokaryotic) and 18S (eukaryotic) ribosomal RNA have resulted in the proposal of a universal phylogenetic tree of life on earth.



Differences between Prokaryotic and Eukaryotic cells

Features	Prokaryotic	Eukaryotic
Size	Size of cell is 1-2µm by 1-4µm or less.	Greater than 5 µm in diameter.
Cell type	Mostly unicellular(some cyanobacteria may be multicellular).	Mostly multicellular.
Nucleus	True nucleus is absent. Nucleus lack nuclear membrane and nucleolus. Such nucleus is called nucleoid.	Nuclear membrane and nucleolus are present.
Chromosome	Usually single circular without histones.	Multiple linear with histones.
Genes	Expressed in groups called operons.	Expressed individually.
Zygote	Merozygotic (partially diploid).	Diploid.

Cell division	Binary fission of budding	Involves mitosis.
Sexual reproduction	No meiosis. Transfer of DNA only.	Involves meiosis.
Permeability of nuclear membrane	Absent.	Selective.
Cytoplasmic streaming	Absent	Present
Cytoskeleton	Absent	Present
Pinocytosis	Absent	Present
Gas vacuoles	Can be present	Absent
Mesosome	Present. Performs the function of Golgi bodies and mitochondria and also help in the separation of chromosome during cell division.	Absent
Ribosome	Smaller size 70S, distributed in the cytoplasm.	Larger size 80s, found on membranes as in endoplasmic reticulum; 70s

		present in organelles such as chloroplast and mitochondria.
Mitochondria	Absent	Present
Chloroplast	Absent	Present
Endoplasmic Reticulum	Absent	Present
Golgi structure	Absent	Present
Membrane bound vacuoles	Absent	Present
Lysosomes and peroxisomes	Absent	Present
Microtubules	Absent or rare	Present
Flagella	Simple structure composed of protein, flagellin.	Complex with 9+2 structure of tubulin and other protein.
Plasma membrane	Generally lack sterol and no carbohydrate. Contain part of respiration	Sterol and carbohydrate is present that serve as receptors. Do not carry out respiration

	and in some photosynthetic machinery.	and photosynthesis.
Glycocalyx	Present as a capsule or slime layer.	Present in some cells that lack cell wall.
Cell wall	Usually present. Chemically complex (typical bacterial cell wall includes peptidoglycan).	When present, chemically simple (includes cellulose and chitin).
Extrachromosomal plasmid	Present. Nonessential prokaryotic genes are encoded on extra chromosomal plasmid.	Absent
Transcription and translation	Occur simultaneously.	Transcription occurs in nucleus and then translation occurs in cytoplasm.
Respiration	Many strict anaerobes.	All aerobic, but some facultative anaerobes by secondary modification.
Photosynthetic enzymes	Bound to plasma membrane as composite chromatophores.	Enzymes packed in plastids bound by membrane.
Nitrogen fixation	Some possess this ability.	None possess this ability.

Metabolic mechanism	Wide variation	Glycolysis, electron transport chain, Krebs cycle.
Duration of cell cycle	Short, takes 20-60 minutes to complete.	Long, takes 12-24 hours to complete.
DNA base ratio as mol% of Guanine+ Cytosine (G+C %)	28-73	About 40

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SCHOOL OF BIO AND CHEMICAL ENGINEERING

DEPARTMENT OF BIOTECHNOLOGY

B.SC- MICROBIOLOGY

UNIT II- GENERAL MICROBIOLOGY - SMB1101

Working Principle and Parts of a Compound Microscope:

The most commonly used microscope for general purposes is the standard compound microscope. It magnifies the size of the object by a complex system of lens arrangement.

It has a series of two lenses; (i) the objective lens close to the object to be observed and (ii) the ocular lens or eyepiece, through which the image is viewed by eye. Light from a light source (mirror or electric lamp) passes through a thin transparent object ().

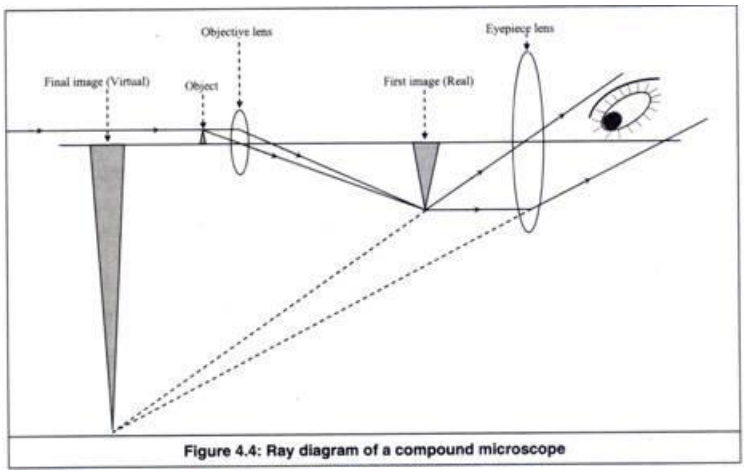


Figure 4.4: Ray diagram of a compound microscope

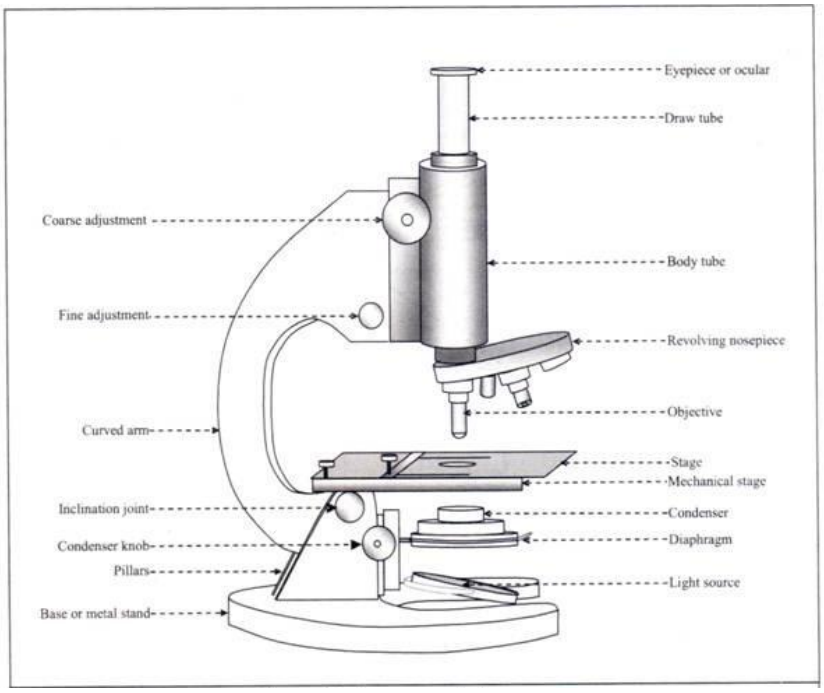
The objective lens produces a magnified 'real image' first image) of the object. This image is again magnified by the ocular lens (eyepiece) to obtain a magnified 'virtual image' (final image), which can be seen by eye through the eyepiece. As light passes directly from the source to the eye through the two lenses, the field of vision is brightly illuminated. That is why; it is a bright-field microscope.

Parts of a Compound Microscope:

The parts of a compound microscope are of two categories as given below:

(i) Mechanical Parts:

These are the parts, which support the optical parts and help in their adjustment for focusing the object.



1. Base or Metal Stand:

The whole microscope rests on this base. Mirror, if present, is fitted to it.

2. Pillars:

It is a pair of elevations on the base, by which the body of the microscope is held to the base

3. Inclination joint:

It is a movable joint, through which the body of the microscope is held to the base by the pillars. The body can be bent at this joint into any inclined position, as desired by the observer, for easier observation. In new models, the body is permanently fixed to the base in an inclined position, thus needing no pillar or joint.

4. Curved Arm:

It is a curved structure held by the pillars. It holds the stage, body tube, fine adjustment and coarse adjustment.

5. Body Tube:

It is usually a vertical tube holding the eyepiece at the top and the revolving nosepiece with the objectives at the bottom. The length of the draw tube is called ‘mechanical tube length’ and is usually 140-180 mm (mostly 160 mm).

6. Draw Tube:

It is the upper part of the body tube, slightly narrower, into which the eyepiece is slipped during observation.

7. Coarse Adjustment:

It is a knob with rack and pinion mechanism to move the body tube up and down for focusing the object in the visible field. As rotation of the knob through a small angle moves the body tube through a long distance relative to the object, it can perform coarse adjustment. In modern microscopes, it moves the stage up and down and the body tube is fixed to the arm.

8. Fine Adjustment:

It is a relatively smaller knob. Its rotation through a large angle can move the body tube only through a small vertical distance. It is used for fine adjustment to get the final clear image. In modern microscopes, fine adjustment is done by moving the stage up and down by the fine adjustment.

9. Stage:

It is a horizontal platform projecting from the curved arm. It has a hole at the center, upon which the object to be viewed is placed on a slide. Light from the light source below the stage passes through the object into the objective.

10. Mechanical Stage (Slide Mover):

Mechanical stage consists of two knobs with rack and pinion mechanism. The slide containing the object is clipped to it and moved on the stage in two dimensions by rotating the knobs, so as to focus the required portion of the object.

11. Revolving Nosepiece:

It is a rotatable disc at the bottom of the body tube with three or four objectives screwed to it. The objectives have different magnifying powers. Based on the required magnification, the nosepiece is rotated, so that only the objective specified for the required magnification remains in line with the light path.

(ii) Optical Parts:

These parts are involved in passing the light through the object and magnifying its size.

The components of optical parts include the following:

1. Light Source:

Modern microscopes have in-built electric light source in the base. The source is connected to the mains through a regulator, which controls the brightness of the field. But in old models, a mirror is used as the light source. It is fixed to the base by a binnacle, through which it can be rotated, so as to converge light on the object. The mirror is plane on one side and concave on the other.

It should be used in the following manner:

(a) Condenser Present:

Only plane side of the mirror should be used, as the condenser converges the light rays.

(b) Condenser Absent:

(i) Daylight:

Plane or concave (plane is easier)

(ii) Small artificial light:

High power objective: Plane side

Low power objective: Concave side

2. Diaphragm:

If light coming from the light source is brilliant and all the light is allowed to pass to the object through the condenser, the object gets brilliantly illuminated and cannot be visualized properly. Therefore, an iris diaphragm is fixed below the condenser to control the amount of light entering into the condenser.

3. Condenser:

The condenser or sub-stage condenser is located between the light source and the stage. It has a series of lenses to converge on the object, light rays coming from the light source. After passing through the object, the light rays enter into the objective.

The ‘light condensing’, ‘light converging’ or ‘light gathering’ capacity of a condenser is called ‘numerical aperture of the condenser’. Similarly, the ‘light gathering’ capacity of an objective is called ‘numerical aperture of the objective’. If the condenser converges light in a wide angle, its numerical aperture is greater and vice versa.

If the condenser has such numerical aperture that it sends light through the object with an angle sufficiently large to fill the aperture back lens of the objective, the objective shows its highest numerical aperture (Figure 4.7). Most common condensers have numerical aperture 1.25.

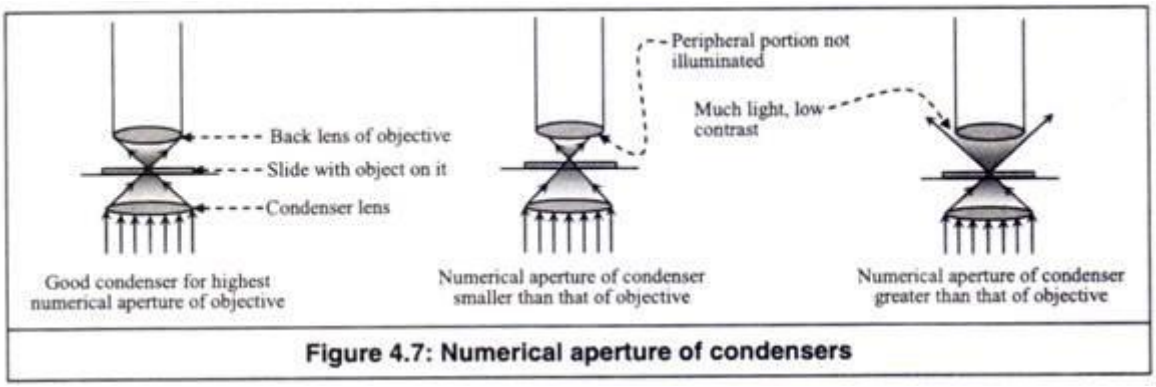


Figure 4.7: Numerical aperture of condensers

If the numerical aperture of the condenser is smaller than that of the objective, the peripheral portion of the back lens of the objective is not illuminated and the image has poor visibility. On the other hand, if the numerical aperture of condenser is greater than that of the objective, the back lens may receive too much light resulting in a decrease in contrast.

There are three types of condensers as follows:

(a) Abbe condenser (Numerical aperture=1.25): It is extensively used.

(b) Variable focus condenser (Numerical aperture =1.25)

(c) Achromatic condenser (Numerical aperture =1.40): It has been corrected for both spherical and chromatic aberration and is used in research microscopes and photomicrographs.

4. Objective:

It is the most important lens in a microscope. Usually three objectives with different magnifying powers are screwed to the revolving nosepiece.

The objectives are:

(a) Low power objective (X 10):

It produces ten times magnification of the object.

(b) High dry objective (X 40):

It gives a magnification of forty times.

(c) Oil-immersion objective (X100):

It gives a magnification of hundred times, when immersion oil fills the space between the object and the objective

The scanning objective (X4) is optional. The primary magnification (X4, X10, X40 or X100) provided by each objective is engraved on its barrel. The oil-immersion objective has a ring engraved on it towards the tip of the barrel.

Resolving Power of Objective:

It is the ability of the objective to resolve each point on the minute object into widely spaced points, so that the points in the image can be seen as distinct and separate from one another, so as to get a clear un-blurred image.

It may appear that very high magnification can be obtained by using more number of high power lenses. Though possible, the highly magnified image obtained in this way is a blurred, one. That means, each point in the object cannot be found as widely spaced distinct and separate point on the image.

Mere increase in size (greater magnification) without the ability to distinguish structural details (greater resolution) is of little value. Therefore, the basic limitation in light microscopes is one not of magnification, but of resolving power, the ability to distinguish two adjacent points as distinct and separate, i.e. to resolve small components in the object into finer details on the image.

Resolving power is a function of two factors as given below:

(a) Numerical aperture (n.a.)

(b) Wavelength of the light (λ)

(a) Numerical aperture:

Numerical aperture is a numerical value concerned with the diameter of the objective lens in relation to its focal length. Thus, it is related to the size of the lower aperture of the objective, through which light enters into it. In a microscope, light is focused on the object as a narrow pencil of light, from where it enters into the objective as a diverging pencil.

The angle 9 subtended by the optical axis (the line joining the centers of all the lenses) and the outermost ray still covered by the objective is a measure of the aperture called 'half aperture angle'.

A wide pencil of light passing through the object 'resolves' the points in the object into widely spaced points on the lens, so that the lens can produce these points as distinct and separate on the image. Here, the lens gathers more light.

On the other hand, a narrow pencil of light cannot 'resolve' the points in the object into widely spaced points on the lens, so that the lens produces a blurred image. Here, the lens gathers less light. Thus, the greater is the width of the pencil of light entering into the objective (29), the higher is its 'resolving power'.

The numerical aperture of an objective is its light gathering capacity, which depends on the site of the angle 8 and the refractive index of the medium existing between the object and the objective.

Numerical aperture (n.a.) = $n \sin \theta$

Where,

n = Refractive index of the medium between the object and the objective and

θ = Half aperture angle

For air, the value of 'n' is 1.00. When the space between the lower tip of the objective and the slide carrying the object is air, the rays emerging through the glass slide into this air are bent or refracted, so that some portion of it do not pass into the objective. Thus, loss of some light rays reduces numerical aperture and decreases the resolving power.

However, when this space is filled with an immersion oil, which has greater refractive index (n=1.56) than that of air (n=1.00), light rays are refracted or bent more towards the objective. Thus, more light rays enter into the objective and greater resolution is obtained. In oil immersion objective, which provides the highest magnification, the size of the aperture is very small.

Therefore, it needs bending of more rays into the aperture, so that the object can be distinctly resolved. That is why, immersion oils, such as cedar wood oil and liquid paraffin are used to fill the gap between the object and the objective, while using oil-immersion objective.

(b) Wavelength of light (λ):

The smaller is the wavelength of light (λ), the greater is its ability to resolve the points on the object into distinctly visible finer details in the image. Thus, the smaller is the wavelength of light, the greater is its resolving power.

Limit of resolution of objective (d):

The limit of resolution of an objective (d) is the distance between any two closest points on the microscopic object, which can be resolved into two separate and distinct points on the enlarged image.

Points with their in-between distance less than ‘d’ or objects smaller than ‘d’ cannot be resolved into separate points on the image. If the resolving power is high, points very close to each other can be seen as clear and distinct.

Thus, the limit of resolution (the distance between the two resolvable points) is smaller. Therefore, smaller objects or finer details can be seen, when’d’ is smaller. Smaller ‘d’ is obtained by increasing the resolving power, which in turn is obtained by using shorter wavelength of light (λ) and greater numerical aperture.

Limit of resolution = $d = \lambda/2$ n.a.

Where,

λ = Wave length of light and

n.a. = Numerical aperture of the objective.

If λ_{green} = 0.55 p and n.a. = 1.30, then d = λ/2 n.a. = 0.55/2 X 1.30 = 0.21 μ. Therefore, the smallest details that can be seen by a typical light microscope is having the dimension of approximately 0.2 μ. Smaller objects or finer details than this cannot be resolved in a compound microscope.

5. Eyepiece:

The eyepiece is a drum, which fits loosely into the draw tube. It magnifies the magnified real image formed by the objective to a still greatly magnified virtual image to be seen by the eye.

Usually, each microscope is provided with two types of eyepieces with different magnifying powers (X10 and X25). Depending upon the required magnification, one of the two eyepieces is inserted into the draw tube before viewing. Three varieties of eyepieces are usually available.

They are the Huygenian, the hyper plane and the compensating. Among them, the Huygenian is very widely used and efficient for low magnification. In this eyepiece, two simple Plano-convex lenses are fixed, one above and the other below the image plane of the real image formed by the objective.

The convex surfaces of both the lenses face downward. The lens towards the objective is called ‘field lens’ and that towards eye, ‘eye lens’. The rays after passing through the eye lens come out through a small circular area known as Rams-den disc or eye point, where the image is viewed by the eye.

Total magnification:

The total magnification obtained in a compound microscope is the product of objective magnification and ocular magnification.

$M_t = M_{ob} \times M_{oc}$

Where,

M_t = Total magnification,

M_{ob} = Objective magnification and

M_{oc} = Ocular magnification

If the magnification obtained by the objective (M_{ob}) is 100 and that by the ocular (M_{oc}) is 10, then total magnification (M_t) = M_{ob} X M_{oc} =100 X 10 =1000. Thus, an object of lq will appear as 1000 µ.

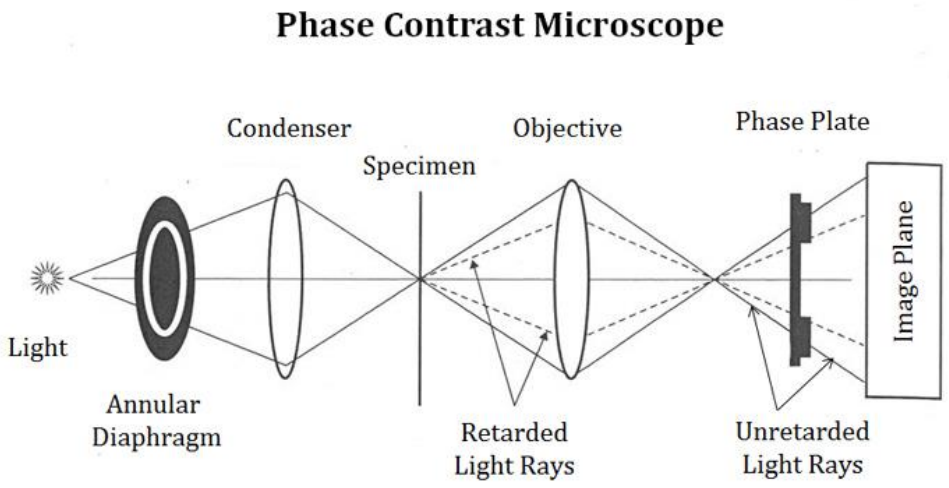
Useful magnification:

It is the magnification that makes visible the smallest resolvable particle. The useful magnification in a light microscope is between X1000 and X2000. Any magnification beyond X2000 makes the image blurred.

Phase contrast microscopy

Unstained living cells absorb practically no light. Poor light absorption results in extremely small differences in the intensity distribution in the image. This makes the cells barely, or not at all, visible in a brightfield microscope. Phase-contrast microscopy is an optical microscopy technique that converts phase shifts in the light passing through a transparent specimen to brightness changes in the image.

- It was first described in 1934 by Dutch physicist Frits Zernike.



When light passes through cells, small phase shifts occur, which are invisible to the human eye. In a phase-contrast microscope, these phase shifts are converted into changes in amplitude, which can be observed as differences in image contrast.

1. Partially coherent illumination produced by the tungsten-halogen lamp is directed through a collector lens and focused on a specialized annulus (labeled condenser annulus) positioned in the substage condenser front focal plane.
2. Wavefronts passing through the annulus illuminate the specimen and either pass through undeviated or are diffracted and retarded in phase by structures and phase gradients present in the specimen.
3. Undeviated and diffracted light collected by the objective is segregated at the rear focal plane by a phase plate and focused at the intermediate image plane to form the final phase-contrast image observed in the eyepieces.

Phase-contrast microscopy is basically a specially designed light microscope with all the basic parts in addition to which an annular phase plate and annular diaphragm are fitted.

The annular diaphragm

- It is situated below the condenser.
- It is made up of a circular disc having a circular annular groove.
- The light rays are allowed to pass through the annular groove.
- Through the annular groove of the annular diaphragm, the light rays fall on the specimen or object to be studied.
- At the back focal plane of the objective develops an image.
- The annular phase plate is placed at this back focal plane.

The phase plate

- It is either a negative phase plate having a thick circular area or a positive phase plate having a thin circular groove.
- This thick or thin area in the phase plate is called the conjugate area.
- The phase plate is a transparent disc.
- With the help of the annular diaphragm and the phase plate, the phase contrast is obtained in this microscope.
- This is obtained by separating the direct rays from the diffracted rays.
- The direct light rays pass through the annular groove whereas the diffracted light rays pass through the region outside the groove.

- Depending upon the different refractive indices of different cell components, the object to be studied shows a different degree of contrast in this microscope.

To produce high-contrast images of transparent specimens, such as

1. living cells (usually in culture),
2. microorganisms,
3. thin tissue slices,
4. lithographic patterns,
5. fibers,
6. latex dispersions,
7. glass fragments, and
8. subcellular particles (including nuclei and other organelles).

Applications of phase-contrast microscopy in biological research are numerous.

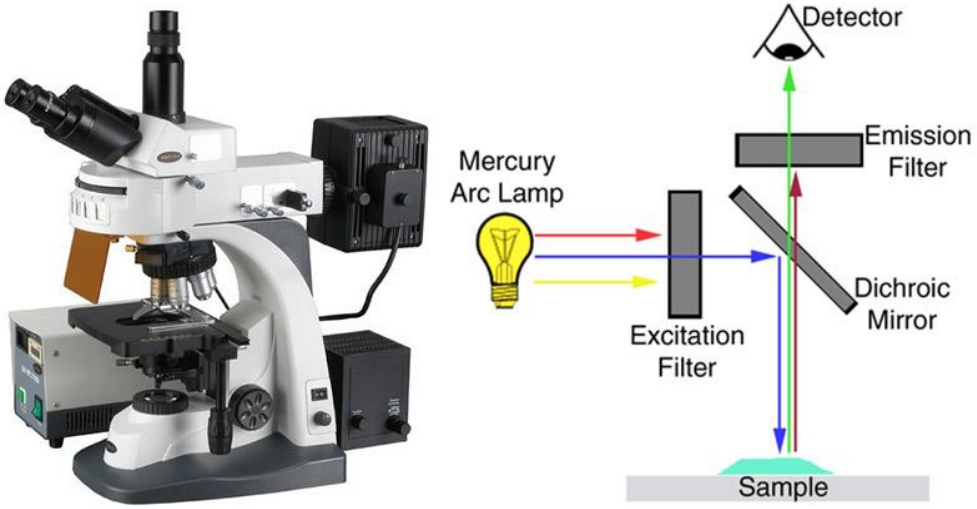
- Living cells can be observed in their natural state without previous fixation or labeling.
- It makes a highly transparent object more visible.
- No special preparation of fixation or staining etc. is needed to study an object under a phase-contrast microscope which saves a lot of time.
- Examining intracellular components of living cells at relatively high resolution. eg: The dynamic motility of **mitochondria**, mitotic chromosomes & vacuoles.
- It made it possible for biologists to study living cells and how they proliferate through cell division.
- Phase-contrast optical components can be added to virtually any brightfield microscope, provided the specialized phase objectives conform to the tube length parameters, and the condenser will accept an annular phase ring of the correct size.

Limitations

- Phase-contrast condensers and objective lenses add considerable cost to a microscope, and so phase contrast is often not used in teaching labs except perhaps in classes in the health professions.
- To use phase-contrast the light path must be aligned.
- Generally, more light is needed for phase contrast than for corresponding bright-field viewing, since the technique is based on the diminishment of the brightness of most objects.

Fluorescence microscope :

- A **fluorescence microscope** is an optical microscope that uses fluorescence and phosphorescence instead of, or in addition to, reflection and absorption to study properties of organic or inorganic substances.
- Fluorescence is the emission of light by a substance that has absorbed light or other electromagnetic radiation while phosphorescence is a specific type of photoluminescence related to fluorescence.
- Unlike fluorescence, a phosphorescent material does not immediately re-emit the radiation it absorbs.
- The fluorescence microscope was devised in the early part of the twentieth century by August Köhler, Carl Reichert, and Heinrich Lehmann, among others.



1. Most cellular components are colorless and cannot be clearly distinguished under a microscope. The basic premise of fluorescence microscopy is to stain the components with dyes.
2. Fluorescent dyes, also known as fluorophores or fluorochromes, are molecules that absorb excitation light at a given wavelength (generally UV), and after a short delay emit light at a longer wavelength. The delay between absorption and emission is negligible, generally on the order of nanoseconds.
3. The emission light can then be filtered from the excitation light to reveal the location of the fluorophores.

- Fluorescence microscopy uses a much higher intensity light to illuminate the sample. This light excites fluorescence species in the sample, which then emit light of a longer wavelength.
- The image produced is based on the second light source or the emission wavelength of the fluorescent species rather than from the light originally used to illuminate, and excite, the sample.

Working

Light of the excitation wavelength is focused on the specimen through the objective lens. The fluorescence emitted by the specimen is focused on the detector by the objective. Since most of the excitation light is transmitted through the specimen, only reflected excitatory light reaches the objective together with the emitted light.

Forms

The "fluorescence microscope" refers to any microscope that uses fluorescence to generate an image, whether it is a more simple set up like an epifluorescence microscope, or a more complicated design such as a confocal microscope, which uses optical sectioning to get better resolution of the fluorescent image.

Most fluorescence microscopes in use are epifluorescence microscopes, where excitation of the fluorophore and detection of the fluorescence are done through the same light path (i.e. through the objective).

Typical components of a fluorescence microscope are:

- **Fluorescent dyes (Fluorophore)**
  - A fluorophore is a fluorescent chemical compound that can re-emit light upon light excitation.
  - Fluorophores typically contain several combined aromatic groups, or plane or cyclic molecules with several π bonds.
  - Many fluorescent stains have been designed for a range of biological molecules.
  - Some of these are small molecules that are intrinsically fluorescent and bind a biological molecule of interest. Major examples of these are nucleic acid stains like DAPI and Hoechst, phalloidin which is used to stain actin fibers in mammalian cells.
- **A light source**

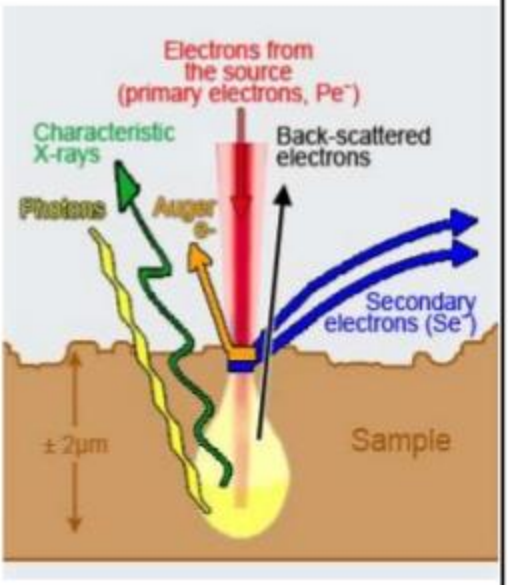
- Four main types of light sources are used, including xenon arc lamps or mercury-vapor lamps with an excitation filter, lasers, and high- power LEDs.
- Lasers are mostly used for complex fluorescence microscopy techniques, while xenon lamps, and mercury lamps, and LEDs with a dichroic excitation filter are commonly used for wide-field epifluorescence microscopes.

- **The excitation filter**
  - The exciter is typically a bandpass filter that passes only the wavelengths absorbed by the fluorophore, thus minimizing the excitation of other sources of fluorescence and blocking excitation light in the fluorescence emission band.
- **The dichroic mirror**
  - A dichroic filter or thin-film filter is a very accurate color filter used to selectively pass light of a small range of colors while reflecting other colors.
- **The emission filter.**
  - The emitter is typically a bandpass filter that passes only the wavelengths emitted by the fluorophore and blocks all undesired light outside this band – especially the excitation light.
  - By blocking unwanted excitation energy (including UV and IR) or sample and system autofluorescence, optical filters ensure the darkest background.
    - To identify structures in fixed and live biological samples.
    - Fluorescence microscopy is a common tool for today's life science research because it allows the use of multicolor staining, labeling of structures within cells, and the measurement of the physiological state of a cell.
    - Fluorescence microscopy is the most popular method for studying the dynamic behavior exhibited in live-cell imaging.
    - This stems from its ability to isolate individual proteins with a high degree of specificity amidst non-fluorescing material.
    - The sensitivity is high enough to detect as few as 50 molecules per cubic micrometer.
    - Different molecules can now be stained with different colors, allowing multiple types of the molecule to be tracked simultaneously.
    - These factors combine to give fluorescence microscopy a clear advantage over other optical imaging techniques, for both in vitro and in vivo imaging.

- Fluorophores lose their ability to fluoresce as they are illuminated in a process called photobleaching. Photobleaching occurs as the fluorescent molecules accumulate chemical damage from the electrons excited during fluorescence.
- Cells are susceptible to phototoxicity, particularly with short-wavelength light. Furthermore, fluorescent molecules have a tendency to generate reactive chemical species when under illumination which enhances the phototoxic effect.
- Unlike transmitted and reflected light microscopy techniques fluorescence microscopy only allows observation of the specific structures which have been labeled for fluorescence.

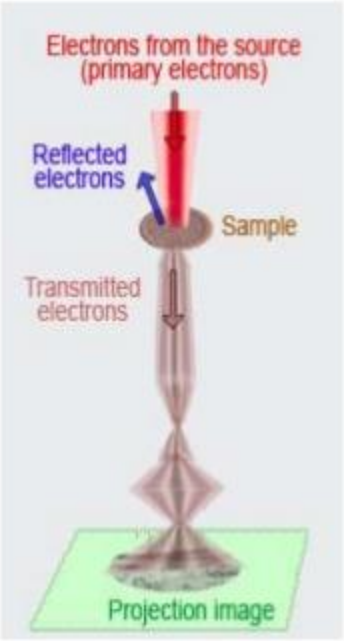
PRINCIPLE OF WORKING OF SEM

- Incoming (primary) electrons
  - can be "reflected" (backscattered) from a bulk specimen.
  - can release secondary electrons.
- Primary electrons are focused into a small-diameter electron probe that is scanned across the specimen.
- Electrostatic or magnetic fields, applied at right angles to the beam, can be used to change its direction of travel.
- By scanning simultaneously in two perpendicular directions, a square or rectangular area of specimen (known as a **raster**) can be covered.
- Image of this area can be formed by collecting secondary electrons from each point on the specimen.



PRINCIPLE OF WORKING OF TEM

- Electrons possess a wave like character.
- Electrons emitted into vacuum from a heated filament with increased accelerating potential will have small wavelength.
- Such higher-energy electrons can penetrate distances of several microns into a solid.
- If these transmitted electrons could be focused - images with much better resolution.
- Focusing relies on the fact that, electrons also behave as negatively charged particles and are therefore deflected by electric or magnetic fields.



Primary Structure of Biological Macromolecules Determines Function

Procaryotic structural components consist of macromolecules such as DNA, RNA, proteins, polysaccharides, phospholipids, or some combination thereof. The macromolecules are made up of primary subunits such as nucleotides, amino acids and sugars (Table 1). It is the sequence in which the subunits are put together in the macromolecule, called the **primary structure**, that determines many of the properties that the macromolecule will have. Thus, the genetic code is determined by specific nuleotide base sequences in chromosomal DNA; the amino acid sequence in a protein determines the properties and function of the protein; and sequence of sugars in bacterial lipopolysaccharides determines unique cell wall properties for pathogens. The primary structure of a macromolecule will drive its function, and differences within the primary structure of biological macromolecules accounts for the immense diversity of life**.**

Macromolecules that make up cell material

Macromolecule	**Primary Subunits**	**Where found in cell**
Proteins	amino acids	Flagella, pili, cell walls, cytoplasmic membranes, ribosomes, cytoplasm
Polysaccharides	sugars (carbohydrates)	capsules, inclusions (storage), cell walls
Phospholipids	fatty acids	membranes
Nucleic Acids (DNA/RNA)	nucleotides	DNA: nucleoid (chromosome), plasmids rRNA: ribosomes; mRNA, tRNA: cytoplasm

Procaryotic Cell Architecture

At one time it was thought that bacteria and other procaryotes were essentially "bags of enzymes" with no inherent cellular architecture. The development of the electron microscope in the 1950s revealed the distinct anatomical features of bacteria and confirmed the suspicion that they lacked a nuclear membrane. Procaryotes are cells of relatively simple construction, especially if compared to eucaryotes. Whereas eucaryotic cells have a preponderance of organelles with separate cellular functions, procaryotes carry out all cellular functions as individual units.

A procaryotic cell has five essential structural components: a **nucleoid (DNA)**, **ribosomes**, **cell membrane**, **cell wall**, and some sort of **surface layer**, which may or may not be an inherent part of the wall.

Structurally, there are three architectural regions: **appendages** (attachments to the cell surface) in the form of **flagella** and **pili (or fimbriae)**; a **cell envelope** consisting of a **capsule**, **cell wall** and **plasma membrane**; and a **cytoplasmic region** that contains the cell **chromosome** (**DNA**) and **ribosomes** and various sorts of **inclusions** (Figure 1).

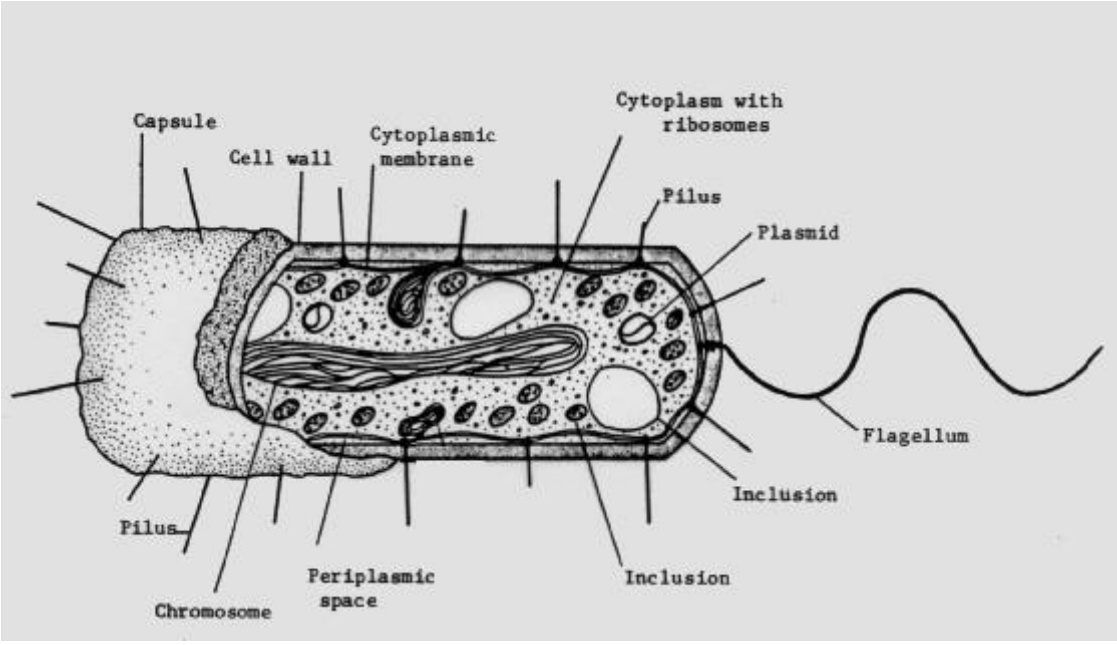


Figure 1. Cutaway drawing of a typical bacterial cell illustrating structural components. See Table below for chemical composition and function of the labeled components.

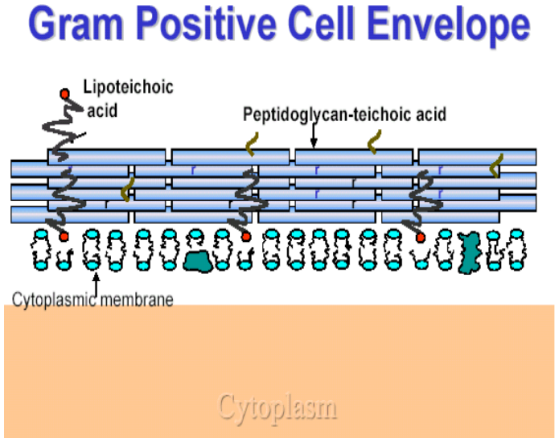
Summary of characteristics of typical bacterial cell structures

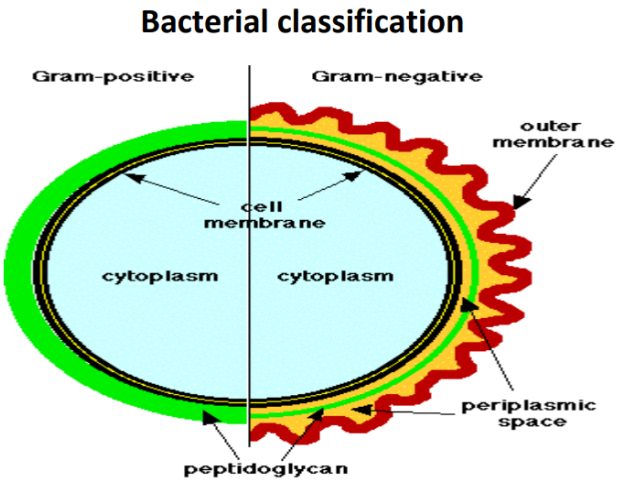
Structure Flagella	Function(s) Swimming movement	Predominant chemical composition Protein
Pili		
Sex pilus	Stabilizes mating bacteria during DNA transfer by conjugation	Protein
Common pili or fimbriae	Attachment to surfaces; protection against phagotrophic engulfment	Protein
Capsules (includes "slime layers" and glycocalyx)	Attachment to surfaces; protection against phagocytic engulfment, occasionally killing or digestion; reserve of nutrients or protection against desiccation	Usually polysaccharide; occasionally polypeptide
Cell wall		
Gram-positive bacteria	Prevents osmotic lysis of cell protoplast and confers rigidity and shape on cells	Peptidoglycan (murein) complexed with teichoic acids
Gram-negative bacteria	Peptidoglycan prevents osmotic lysis and confers rigidity and shape; outer membrane is permeability barrier; associated LPS and proteins have various functions	Peptidoglycan (murein) surrounded by phospholipid protein-lipopolysaccharide "outer membrane"
Plasma membrane	Permeability barrier; transport of solutes; energy generation; location of numerous enzyme systems	Phospholipid and protein
Ribosomes	Sites of translation (protein synthesis)	RNA and protein
Inclusions	Often reserves of nutrients; additional specialized functions	Highly variable; carbohydrate, lipid, protein or inorganic

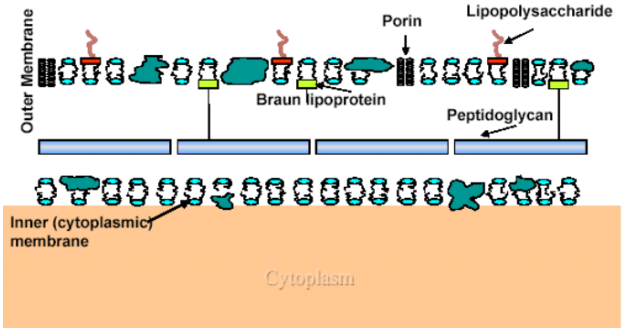
Chromosome	Genetic material of cell	DNA
Plasmid	Extrachromosomal genetic material	DNA

Gram-Positive bacteria	Gram-Negative bacteria
Cell Wall	
A single-layered, smooth cell wall	A double-layered, wavy cell-wall
Cell Wall thickness	
The thickness of the cell wall is 20 to 80 nanometres	The thickness of the cell wall is 8 to 10 nanometres
Peptidoglycan Layer	
It is a thick layer/ also can be multilayered	It is a thin layer/ often single-layered.
Teichoic acids	
Presence of teichoic acids	Absence of teichoic acids
Outer membrane	
The outer membrane is absent	The outer membrane is present (mostly)
Porins	
Absent	Occurs in Outer Membrane

Morphology	
Cocci or spore-forming rods	Non-spore forming rods.
Flagella Structure	
Two rings in basal body	Four rings in basal body
Lipid content	
Very low	20 to 30%
Lipopolysaccharide	
Absent	Present
Toxin Produced	
Exotoxins	Endotoxins or Exotoxins
Resistance to Antibiotic	
More susceptible	More resistant
Examples	
Staphylococcus, Streptococcus, etc.	Escherichia, Salmonella, etc.







The simple stain:

The simple stain can be used as a quick and easy way to determine cell shape, size and arrangements of bacteria. True to its name, the simple stain is a very simple staining procedure involving single solution of stain. Any basic dye such as methylene blue, safranin, or crystal violet can be used to color the bacterial cells.

These stains will readily give up a hydroxide ion or accept a hydrogen ion, which leaves the stain positively charged. Since the surface of most bacterial cells and cytoplasm is negatively charged, these positively charged stains adhere readily to the cell surface. After staining, **bacterial cell morphology** (shape and arrangements) can be appreciated.

Preparation of a smear and heat fixing

1. Using a sterilized inoculating loop, transfer loopful of liquid suspension containing bacteria to a slide (clean grease free microscopic slide) or transfer an isolated colony from a culture plate to a slide with a water drop.
2. Disperse the bacteria on the loop in the drop of water on the slide and spread the drop over an area the size of a dime. It should be a thin, even smear.
3. Allow the smear to dry thoroughly.
4. Heat-fix the smear cautiously by passing the underside of the slide through the burner flame two or three times. It fixes the cell in the slide. Do not overheat the slide as it will distort the bacterial cells.

Staining

1. Cover the smear with methylene blue and allow the dye to remain in the smear for approximately one minute (Staining time is not critical here; somewhere between 30 seconds to 2 minutes should give you an acceptable stain, the longer you leave the dye in it, the darker will be the stain).
2. Using distilled water wash bottle, gently wash off the excess methylene blue from the slide by directing a gentle stream of water over the surface of the slide.
3. Wash off any stain that got on the bottom of the slide as well.

4. Saturate the smear again but this time with Iodine. Iodine will set the stain
5. Wash of any excess iodine with gently running tap water. Rinse thoroughly. *(You may not get mention about step 4 and 5 in some text books)*
6. Wipe the back of the slide and blot the stained surface with bibulous paper or with a paper towel.
7. Place the stained smear on the microscope stage smear side up and focus the smear using the 10X objective.
8. Choose an area of the smear in which the cells are well spread in a monolayer. Center the area to be studied, apply immersion oil directly to the smear, and focus the smear under oil with the 100X objective.
9. The bacterial cells usually stain uniformly and the color of the cell depends on the type of dye used. If methyene blue is used, some granules in the interior of the cells of some bacteria may appear more deeply stained than the rest of the cell, which is due to presence of different chemical substances.

Uses: Diagnostic microbiology laboratory generally does not perform simple staining method. Differential staining such as **Gram Staining** and **AFB Staining** are commonly used to identify and differentiate the bacterial isolates. Simple staining can be useful in the following circumstances.

1. To differentiate bacteria from yeast cells: When endocervical swab culture is done in Blood agar both *Staphylococcus* spp and yeast cells may give similar looking colonies in Blood agar (a common error for new technologist or microbiologist with less experience). Performing wet mount technique or simple staining from the isolate can be helpful.
2. To presumptively identify the bacterial isolate
   Due to their ubiquitous presence, *Bacillus* spp may present as a contaminant in the culture plates. In some circumstances (e.g. growth in **Blood Agar** but no growth in **MacConkey Agar**), identifying the shape of the bacteria (rod or cocci) may help to eliminate the isolate as possible contaminants (e.g., *Bacillus* spp) or further process as potential pathogen (cocci).

Grams staining

The two major groups of bacteria can be divided into gram-positive and gram-negative. The Gram stain technique is based on the differential structure of the cellular membranes and cell walls of the two groups.

Gram-positive organisms contain a highly cross-linked layer of peptidoglycan that retains the primary dye, crystal violet (CV), following the application of the mordant, iodine (I). The iodine and crystal violet form a complex within the peptidoglycan. When decolorizer is applied to the cells, the CV-I complex remains within the cell, making it appear dark purple to blue.

The gram-negative organisms do not contain a thick cross-linked layer of peptidoglycan. The peptidoglycan is loosely distributed between the inner cell and the outer cell membranes. Following the application of the crystal violet and iodine, the CV-I complexes are not trapped within the peptidoglycan. Application of the acid-alcohol decolorizer dehydrates the outer cellular membrane, leaving holes in the membrane and effectively washing or removing the CV-I complex from the cells. The cells appear colorless. To make the colorless cells visible, a secondary stain, safranin, is applied, leaving the gram-negative cells pink.

1. Prepare and fix the specimen to the microscope slide before staining.
2. Cover the smear with crystal violet, the primary stain, for 20 seconds.
3. Gently rinse off the stain with water.
4. Cover the smear with Gram's iodine, the mordant, for 1 minute.
5. Pour off the excess Gram's iodine.
6. Run the acid-alcohol decolorizer over the smear until the solution appears clear.
7. Gently rinse with water.
8. Cover the smear with safranin, the secondary or counterstain, for 20 seconds.
9. Gently rinse the stain with water.
10. Blot dry with bibulous paper.
11. Over-decolorization may result in the identification of false gram-negative results, whereas under-decolorization may result in the identification of false gram-positive results.
12. Smears that are too thick or viscous may retain too much primary stain, making the identification of proper Gram stain reactions difficult. Gram-negative organisms may not decolorize properly.

13. Cultures older than 16 to 18 hours will contain living and dead cells. Cells that are dead will be deteriorating and will not retain the stain properly.
14. The stain may form a precipitate with aging. Filtering through gauze will remove excess crystals.
15. Gram stains from patients on antibiotics or antimicrobial therapy may have altered Gram stain reactivity due to the successful treatment.
16. Occasionally, pneumococci identified in the lower respiratory tract on a direct smear will not grow in culture. Some strains are obligate anaerobes.
17. Toxin-producing organisms such as Clostridia, staphylococci, and streptococci may destroy white blood cells within a purulent specimen.
18. Faintly staining Gram-negative organisms, such as *Campylobacter* and *Brucella*, may be visualized using an alternative counterstain (e.g., basic fuchsin).

Examples
Gram-Positive: *Streptococcus, Staphylococcus, Corynebacterium, Listeria, Bacillus, Clostridium,* etc.
Gram-Negative: *E. coli, Salmonella Typhi, Shigella spp, Pseudomonas aeruginosa, Neisseria gonorrhoeae, Chlamydia trachomatis, Yersinia pestis*, etc.

Acid-fast staining

Acid-fast mycobacteria contain mycolic acid in their outer membrane, making the cells waxy and resistant to staining with aqueous based stains such as the Gram stain. The primary stain, carbolfuchsin is applied to the cells, and heat and phenol are used to allow the stain to penetrate into the waxy surface of acid-fast microorganisms. The excess stain is removed with treatment by acid alcohol (ethanol and hydrochloric acid). A secondary stain, methylene blue, is then applied to the cells.

Primary Stain: 0.3% Carbol-fuchsin. Dissolve 50 g phenol in 100 mL ethanol (95%) or methanol (95%). Dissolve 3 g Basic fuchsin in the mixture and add distilled water to bring the volume to 1 L.

Decolorization Solution: Add 30 mL hydrochloric acid to 1 L of 95% denatured alcohol. Cool and mix well before use. Alternate decolorizing reagent (without alcohol): Slowly add 250 mL sulfuric acid (at least 95%) to 750 mL distilled water. Cool and mix well before using. **Counterstain:** 0.3% nethylene blue. Dissolve 3 g nethylene blue in 1 L distilled water.

1. Prepare and fix the specimen smear prior to staining.
2. Place a small strip of blotting or filter paper over the top of the specimen, and place the slide over a boiling hot water bath on a mesh surface.
3. Cover the filter paper with the primary stain, carbolfuchsin. Leave the slide on the water bath for 3 to 5 minutes. Continue to apply stain if the filter paper begins to dry.
4. Remove the filter paper and rinse the slide with water until the solution runs clear.
5. Run acid-alcohol decolorizer over the slide for approximately 10 to 15 seconds.
6. Rinse the slide with water.
7. Cover the smear with the secondary or counterstain, methylene blue, for 1 minute.
8. Gently rinse the slide with water.
9. Blot the slide dry with bibulous paper.

Acid fast: Bright red to intensive purple, Red, straight or slightly curved rods, occurring singly or in small groups, may appear beaded

Non-acid fast: Blue color; In addition, background material should stain blue.

1. The filter paper must remain moist and in contact with the specimen during heating to allow for proper penetration of the primary stain.
2. Organisms cultivated on blood agar may experience nutrient deprivation, resulting in a lower lipid content in the outer membrane resulting in poor staining.

Examples
Acid-fast: *Mycobacterium tuberculosis, Mycobacterium smegmatis*.
Non-Mycobacterial bacteria: *Nocardia*
Coccidian Parasites: *Cryptosporidium*

Capsule Stain-

A capsule is a gelatinous outer layer that is secreted by the cell and that surrounds and adheres to the cell wall. It is not common to all organisms. Cells that have a heavy capsule are generally virulent and capable of producing disease, since the structure protects bacteria against the normal phagocytic activities of host cells. Chemically, the capsular material is composed mainly of complex polysaccharides such as levans, dextrans, and celluloses.

Capsule staining is more difficult than other types of differential staining procedures because the capsular materials are water-soluble and may be dislodged and removed with vigorous washing. Smears should not be heated because the resultant cell shrinkage may create a clear zone around the organism that is an artifact that can be mistaken for the capsule.

1. To prepare a smear of an encapsulated bacterium and stain its capsule using the Anthony capsule stain.
2. To visualize the capsule and differentiate it from the cell body.

Primary Stain: Crystal Violet (1% aqueous)

A violet stain is applied to a non–heat-fixed smear. At this point, the cell and the capsular material will take on the dark color.

Decolorizing Agent: Copper Sulfate (20%)

Because the capsule is nonionic, unlike the bacterial cell, the primary stain adheres to the capsule but does not bind to it. In the capsule staining method, copper sulfate is used as a decolorizing agent rather than water. The copper sulfate washes the purple primary stain out of the capsular material without removing the stain bound to the cell wall. At the same time, the decolorized capsule absorbs the copper sulfate, and the capsule will now appear blue in contrast to the deep purple color of the cell.

1. Prepare thin smears of bacterial culture on a microscope slide.
2. Allow the smear to only air-dry. *Do not heat-fix as this will cause the capsule to shrink or be destroyed.*

3. Apply 1% crystal violet and allow it to remain on the slide for 2 minutes.
4. With the slide over the proper waste container provided, gently wash off the crystal violet with 20% copper sulfate. *Caution: Do not wash the copper sulfate and stain directly into the sink.*
5. Blot the slide dry with bibulous paper.
6. Observe with the oil immersion lens.

Capsulated: *Bacillus anthracis, Klebsiella pneumoniae, Streptococcus pneumonia, Neisseria meningitidis Clostridium spp,* etc.
Non-Capsulated: *Neisseria gonorrhoreae,* etc.

Spore Stain-

Members of the anaerobic genera *Clostridium* and *Desulfotomaculum* and the aerobic genus *Bacillus* are examples of organisms that have the capacity to exist either as metabolically active vegetative cells or as highly resistant, metabolically inactive cell types called spores. When environmental conditions become unfavorable for continuing vegetative cellular activities, particularly with the exhaustion of a nutritional carbon source, these cells have the capacity to undergo sporogenesis and give rise to a new intracellular structure called the endospore, which is surrounded by impervious layers called spore coats. As conditions continue to worsen, the endospore is released from the degenerating vegetative cell and becomes an independent cell called a free spore. Because of the chemical composition of spore layers, the spore is resistant to the damaging effects of excessive heat, freezing, radiation, desiccation, and chemical agents, as well as to the commonly employed microbiological stains. With the return of favorable environmental conditions, the free spore may revert to a metabolically active and less resistant vegetative cell through germination. It should be emphasized that sporogenesis and germination are not means of reproduction but merely mechanisms that ensure cell survival under all environmental conditions.

In practice, the spore stain uses two different reagents. An alternative method known as the Dorner method is widely published and utilizes nigrosin as the counterstain.

1. To prepare an endospore stain of bacterial cells and demonstrate endospores in the stained preparation.

2. To differentiate between vegetative cells and endospores.
    3. **Malachite Green:** Unlike most vegetative cell types that stain by common procedures, the free spore, because of its impervious coats, will not accept the primary stain easily. For further penetration, the application of heat is required. After the primary stain is applied and the smear is heated, both the vegetative cell and spore will appear green.
    4. **Decolorizing Agent:** Water Once the spore accepts the malachite green, it cannot be decolorized by tap water, which removes only the excess primary stain. The spore remains green. On the other hand, the stain does not demonstrate a strong affinity for vegetative cell components; the water removes it, and these cells will be colorless.
    5. **Counterstain (Safranin):** This contrasting red stain is used as the second reagent to color the decolorized vegetative cells, which will absorb the counterstain and appear red. The spores retain the green of the primary stain.
    6. Prepare smears in the usual manner using aseptic technique.
    7. Allow smear to air-dry, and heat fix in the usual manner.
    8. Flood smears with malachite green and place on top of a beaker of water sitting on a warm hot plate, allowing the preparation to steam for 2 to 3 minutes. *Note: Do not allow stain to evaporate; replenish stain as needed. Prevent the stain from boiling by adjusting the hot plate temperature.*
    9. Remove slides from hot plate, cool, and wash under running tap water.
    10. Counterstain with safranin for 30 seconds.
    11. Wash with tap water.
    12. Blot dry with bibulous paper and examine under oil immersion.

Positive: *Clostridium perfringens, C. botulinum, C. tetani, Bacillus anthracis, Bacillus cereus, Desulfotomaculum* spp, *Sporolactobacillus spp, Sporosarcina* spp, etc.
Negative: *E. coli, Salmonella* spp, etc.
Endospores: Endospores are bright green.
Vegetative Cells: Vegetative cells are brownish red to pink.

Lactophenol Cotton Blue:

Lactophenol Cotton Blue (LPCB) Staining method works on the principle of aiding the identification of the fungal cell walls.

- Fungi are eukaryotic organisms with both macroscopic and microscopic characteristics.
- The fungal spore cell wall is made up of chitin of which the components of the Lactophenol Cotton Blue solution stains for identification.
- The lactophenol cotton blue solution acts as a mounting solution as well as a staining agent.
- The solution is clear and blue in color and it is made up of a combination of three main reagents:
  - **Phenol**: It acts as a disinfectant by killing any living organisms
  - **Lactic acid**: To preserve the fungal structures
  - **Cotton blue**: To stain or give color to the chitin on the fungal cell wall and other fungal structures
- The stain will give the fungi a blue-colored appearance of the fungal spores and structures, such as hyphae.

Lactophenol Cotton Blue solution is prepared for over two days leaving the reagents undisturbed to allow dissolving and maturation.

1. Day 1: Dissolve the cotton blue in distilled water and leave to rest overnight. This eliminates insoluble dye.
2. Day2: Using protective gloves, add phenol crystals to lactic acid in a glass beaker and stir using a magnetic stirrer until the crystals dissolve.
3. Add glycerol
4. Filter the Cotton blue and the distilled water into the phenol + glycerol +lactic acid solution and mix.
5. Store at room temperature.
6. On a clean microscopic glass slide, add a drop of 70% ethanol
7. Add the fungal specimen to the drop of alcohol using a sterile mounter such as an inoculation loop (from solid medium), depending on the sample of use.
8. Tease the fungal sample of the alcohol using a needle mounter, to ensure the sample mixes well with the alcohol.

9. Using a dropper or pipette, add one or two drops of Lactophenol Cotton Blue Solution (prepared above) before the ethanol dries off.
10. Carefully cover the stain with a clean sterile coverslip without making air bubbles to the stain.
11. Examine the stain microscopically at 40X, to observe for fungal spores and other fungal structures.

Fungal spores, hyphae, and fruiting structures stain blue while the background stains pale blue.

For example,

- *Aspergillus niger* stains the hyphae and fruiting structures a delicate blue with a pale blue background.
- *Trichophyton mentagrophytes* also stains the hyphae and fruiting structures a delicate blue with a pale blue background.
- It can only be used as a presumptive identification method of fungi which should be followed up with other diagnostic tools such as biochemical and cultural examination.
- The components of the solution should be used before expiry, including the use of the solution before it expires.
- The solution may disrupt the original morphology of the fungi.
- The stain can only be used to identify mature fungi and its structures and not the young vegetative forms of fungi.
- The stain can not be stored for a long period of time.

Applications

- Used in the identification of suspected fungal samples.
- General identification of fungi and its structures.

KOH MOUNT:

A KOH test may be recommended for someone who has symptoms of a fungal infection of the skin, hair, or nails, such as:

- A localized rash
- Ring-shaped, red patches with scaly edges
- Severe itching of the skin[2]
- Rashes that fail to respond to or worsen with corticosteroid medications
- Rashes that persist for months

A KOH test can confirm the presence of fungi, including dermatophytes. Dermatophytes are fungi that require keratin for growth. Diseases caused by dermatophytes include athlete's foot, jock itch, nail infections, and ringworm.[3] They commonly cause skin infections of the feet, the genitals, and, particularly in kids, the scalp.[4]

The KOH test may be used after a clinical examination and a Wood lamp examination, which uses ultraviolet light to look closely at the skin.[5]

KOH prep tests are not done for every rash. Instead, they are typically ordered when there's a question as to the cause of the rash.

Risks and Contraindications

The KOH prep test carries little risk aside from a small chance of bleeding or infection as a result of scraping the skin to obtain a sample.[1]

After the test, your doctor will provide instructions for how to care for the area from which the sample was taken.

Call your doctor know if you develop signs of infection in the scraped area, including:

- Increased redness
- Swelling

- Heat
- Pain

There are no specific contraindications for this test.

During the Test

If you see your doctor for a rash that they suspect upon visual examination might be due to a fungal infection, they will likely do a KOH prep test on the spot in the office. You will not have to make a separate appointment or do any type of preparation beforehand.

The test itself will take no more than a few minutes and will proceed as follows:

The affected skin or nail is gently scraped with a small scalpel or the edge of a glass slide. The scrapings from the skin are placed on a microscope slide and a few drops of a potassium hydroxide (KOH) solution are added. The slide is heated for a short time and then examined under the microscope.

Potassium hydroxide (KOH) solution is alkaline and has the ability to dissolve keratin that is scraped from the outer layer of the skin. As the KOH dissolves the material binding the skin cells together, any fungus present is released. This allows for the identification of organisms such as dermatophytes.[6]

You may feel pressure when the doctor or nurse scrapes your skin. In some cases, the scraping may feel slightly uncomfortable if it is taken from under a nail or if the area from where the sample is being obtained is tender. Generally, though, there is no pain associated with the procedure itself.

After the test, the skin may be slightly tender where it was scraped, but this will fade over the course of a few hours. Most people, though, do not notice any after-effects.

Interpreting Results

KOH destroys all non-fungal cells, and so when the liquid is examined under the microscope, your doctor is able to see if there is any fungus present in the liquid.

Normal results indicate that there is no fungus present in your skin sample. This means your skin rash is not being caused by a fungal infection, but rather something else.

Abnormal results mean that fungus is present and your skin rash is being caused by a fungal infection. A KOH test can confirm the presence of dermatophytes, which include epidermophyton, trichophytan, and microsporum. It can also test for *Candida albicans*. This same yeast that causes oral thrush and vaginal infections can cause raised, itchy skin rashes as well.[7]

Common skin conditions that can be found using the KOH prep test include:

- Ringworm
- Athlete's foot
- Jock itch
- Fungal infections of the nail[8]

Your doctor will be able to share the results with you immediately and provide you with treatment options before you leave the appointment.

A KOH test does not specifically identify the type of fungus, only shows that fungus is present. In most cases, it's not necessary to know exactly the type of fungus causing the rash; your dermatologist can make an educated judgment and prescribe treatment.

A fungal culture can be done if your dermatologist decides it is necessary. A fungal culture allows the fungus to grow so that the specific type can be identified. Results of a fungal culture can take weeks, so this test is done only in cases where knowing the specific strain of fungus is necessary to develop a more precise treatment plan.[9]

A skin biopsy may be necessary if the results from the KOH test are inconclusive