# SATHYABAMA

INSTITUTE OF SCIENCE AND TECHNOLOGY
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## SCHOOL OF BIO AND CHEMICAL ENGINEERING

## DEPARTMENT OF BIOTECHNOLOGY

## B.SC. BIOTECHNOLOGY / BIOCHEMISTRY

## UNIT - 1 - FUNDAMENTALS OF MICROBIOLOGY - SBB1102

# History and scope of Microbiology

**Overview**

This chapter introduces the field of microbiology and discusses the importance of microorganisms not only as causative agents of disease but also as important contributors to food production, antibiotic manufacture, vaccine development, and environmental management. It presents a brief history of the science of microbiology, an overview of the microbial world, a discussion of the scope and relevance of microbiology in today's society, and predictions about the future of microbiology.

**Objectives**

After reading this chapter you should be able to:

1. define the science of microbiology and describe some of the general methods used in the study of microorganisms
2. discuss the historical concept of spontaneous generation and the experiments that were performed to disprove this erroneous idea
3. discuss how Koch's postulates are used to establish the causal link between a suspected microorganism and a disease
4. describe some of the various activities of microorganisms that are beneficial to humans
5. describe procaryotic and eucaryotic morphology, the two types of cellular anatomy, and also the distribution of microorganisms among the various kingdoms or domains in which living organisms are categorized
6. discuss the importance of the field of microbiology to other areas of biology and to general human welfare

**Study Outline**

I. Microbiology – An Introduction
   A. Microbiology is the study of organisms too small to be clearly seen by the unaided eye (i.e., microorganisms); these include viruses, bacteria, archaea, protozoa, algae, and fungi
   B. Some microbes (e.g., algae and fungi) are large enough to be visible, but are still included in the field of microbiology; it has been suggested that microbiology be defined not only by the size of the organisms studied but by techniques employed to study them (isolation, sterilization, culture in artificial media)

II. The Discovery of Microorganisms
   A. Invisible living creatures were thought to exist and were thought to be responsible for disease long before they were observed
   B. Antony van Leeuwenhoek (1632-1723) constructed microscopes and was the first person to observe and describe microorganisms accurately

III. The Conflict over Spontaneous Generation
   A. The proponents of the concept of spontaneous generation claimed that living organisms could develop from nonliving or decomposing matter
   B. Francesco Redi (1626-1697) challenged this concept by showing that maggots on decaying meat came from fly eggs deposited on the meat, and not from the meat itself

C. John Needham (1713-1781) showed that mutton broth boiled in flasks and then sealed could still develop microorganisms, which supported the theory of spontaneous generation
D. Lazzaro Spallanzani (1729-1799) showed that flasks sealed and then boiled had no growth of microorganisms, and he proposed that air carried germs to the culture medium; he also commented that external air might be needed to support the growth of animals already in the medium; the latter concept was appealing to supporters of spontaneous generation
E. Louis Pasteur (1822-1895) trapped airborne organisms in cotton; he also heated the necks of flasks, drawing them out into long curves, sterilized the media, and left the flasks open to the air; no growth was observed because dust particles carrying organisms did not reach the medium, instead they were trapped in the neck of the flask; if the necks were broken, dust would settle and the organisms would grow; in this way Pasteur disproved the theory of spontaneous generation
F. John Tyndall (1820-1893) demonstrated that dust did carry microbes and that if dust was absent, the broth remained sterile-even if it was directly exposed to air; Tyndall also provided evidence for the existence of heat-resistant forms of bacteria

IV. The Role of Microorganisms in Disease
A. Recognition of the relationship between microorganisms and disease
1. Agostino Bassi (1773-1856) showed that a silkworm disease was caused by a fungus
2. M. J. Berkeley (ca. 1845) demonstrated that the Great Potato Blight of Ireland was caused by a fungus
3. Louis Pasteur showed that the péine disease of silkworms was caused by a protozoan parasite
4. Joseph Lister (1872-1912) developed a system of surgery designed to prevent microorganisms from entering wounds; his patients had fewer postoperative infections, thereby providing indirect evidence that microorganisms were the causal agents of human disease; his published findings (1867) transformed the practice of surgery
5. Robert Koch (1843-1910), using criteria developed by his teacher, Jacob Henle (1809-1895), established the relationship between Bacillus anthracis and anthrax; his criteria became known as Koch's Postulates and are still used to establish the link between a particular microorganism and a particular disease:
a. The microorganisms must be present in every case of the disease but absent from healthy individuals
b. The suspected microorganisms must be isolated and grown in pure culture
c. The same disease must result when the isolated microorganism is inoculated into a healthy host
d. The same microorganism must be isolated again from the diseased host
6. Koch's work was independently confirmed by Pasteur
B. The development of techniques for studying microbial pathogens

1. Koch and his associates developed techniques, reagents, and other materials for culturing bacterial pathogens on solid growth media; these enable microbiologists to isolate microbes in pure culture
2. Charles Chamberland [1851-1908] constructed a bacterial filter that removed bacteria and larger microbes from specimens; this led to the discovery of viruses as disease-causing agents

C. Immunological studies
1. Edward Jenner [ca. 1798] used a vaccination procedure to protect individuals from smallpox
2. Louis Pasteur developed other vaccines including those for chicken cholera, anthrax, and rabies
3. Emil von Behring (1854-1917) and Shibasaburo Kitasato (1852-1931) induced the formation of diphtheria toxin antitoxins in rabbits; the antitoxins were effectively used to treat humans and provided evidence for humoral immunity
4. Elie Metchnikoff (1845-1916) demonstrated the existence of phagocytic cells in the blood, thus demonstrating cell-mediated immunity

V. Industrial Microbiology and Microbial Ecology
A. Louis Pasteur demonstrated that alcoholic fermentations were the result of microbial activity, that some organisms could decrease alcohol yield and sour the product, and that some fermentations were aerobic and some anaerobic; he also developed the process of pasteurization to preserve wine during storage
B. Sergei Winogradsky (1856-1953) worked with soil bacteria and discovered that they could oxidize iron, sulfur, and ammonia to obtain energy; he also studied anaerobic nitrogen fixation and cellulose decomposition
C. Martinus Beijerinck (1851-1931) isolated aerobic nitrogen-fixing bacteria, a root-nodule bacterium capable of fixing nitrogen, and sulfate reducing bacteria
D. Beijerinck and Winogradsky pioneered the use of enrichment cultures and selective media

VI. The Members of the Microbial World
A. Procaryotes have a relatively simple morphology and lack a true membrane-delimited nucleus
B. Eucaryotes are morphologically complex and have a true, membrane-enclosed nucleus
C. In a commonly used classification scheme, organisms are divided into five kingdoms: the Monera or Procaryotae, Protista, Fungi, Animalia, and Plantae; microbiologists are concerned primarily with members of the first three kingdoms and also with viruses, which are not classified with living organisms
D. Recently a classification scheme consisting of three domains (Bacteria, Archaea, and Eucarya) has become widely accepted; this scheme is followed in this textbook

VII. The Scope and Relevance of Microbiology
A. Microorganisms were the first living organisms on the planet, live everywhere life is possible, are more numerous than any other kind of organism, and probably constitute the largest component of the earth's biomass

B. The entire ecosystem depends on the activities of microorganisms, and microorganisms influence human society in countless ways
C. Microbiology has an impact on many fields including medicine, agriculture, food science, ecology, genetics, biochemistry, and molecular biology
D. Microbiologists may be interested in specific types of organisms:
    1. Virologists-viruses
    2. Bacteriologists-bacteria
    3. Phycologists or Algologists-algae
    4. Mycologists-fungi
    5. Protozoologists-protozoa
E. Microbiologists may be interested in various characteristics or activities of microorganisms:
    1. Microbial morphology
    2. Microbial cytology
    3. Microbial physiology
    4. Microbial ecology
    5. Microbial genetics and molecular biology
    6. Microbial taxonomy
F. Microbiologists may have a more applied focus:
    1. Medical microbiology, including immunology
    2. Food and dairy microbiology
    3. Public health microbiology
    4. Agricultural microbiology
    5. Industrial microbiology

VIII. The Future of Microbiology
A. Microbiology has had and will continue to have a profound influence on society.
B. In the future microbiologists will be:
    1. Trying to better understand and control existing, emerging, and reemerging infectious diseases
    2. Studying the association between infectious agents and chronic diseases
    3. Learning more about host defenses and host-pathogen interactions
    4. Developing new uses for microbes in industry, agriculture, and environmental control
    5. Still discovering the many microbes that have not yet been identified and cultured
    6. Trying to better understand how microbes interact and communicate
    7. Analyzing and interpreting the ever-increasing amount of data from genome studies
    8. Continuing to use microbes as model systems for answering fundamental questions in biology
    9. Assessing and communicating the potential impact of new discoveries and technologies on society

## 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.

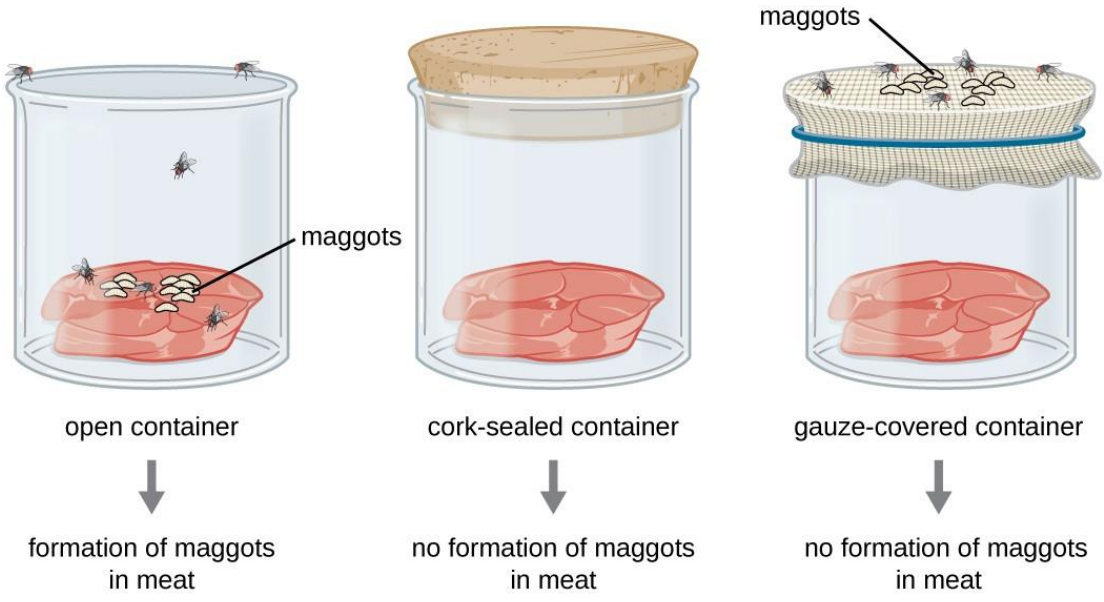


Figure 1. Francesco Redi's experimental setup consisted of an open container, a container sealed with a cork top, and a container covered in mesh that let in air but not flies. Maggots only appeared on the meat in the open container. However, maggots were also found on the gauze of the gauze-covered container.

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.[2] 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.[3] 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).

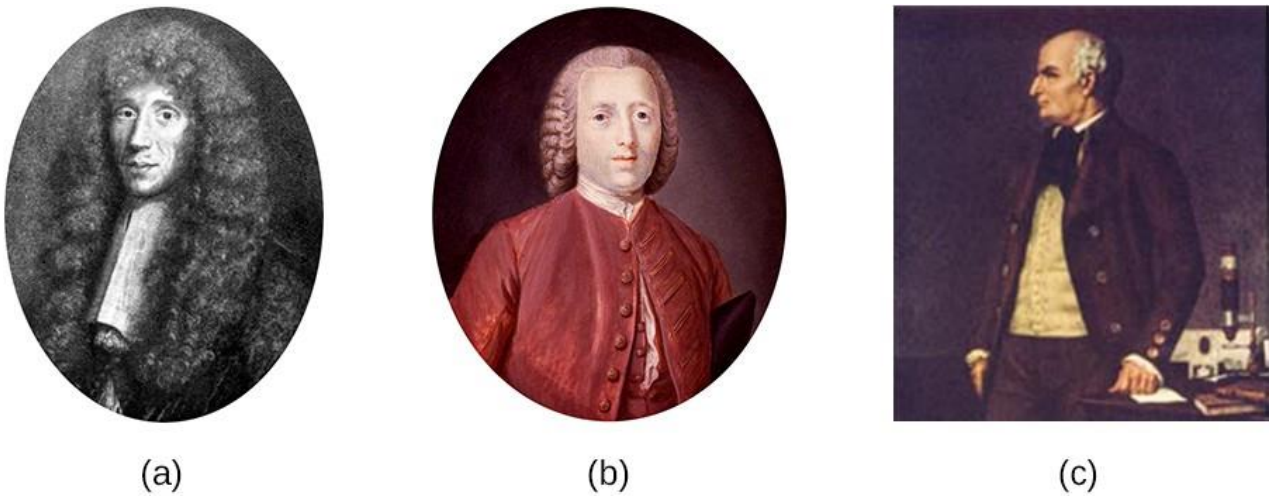



Figure 2. (a) Francesco Redi, who demonstrated that maggots were the offspring of flies, not products of spontaneous generation. (b) John Needham, who argued that microbes arose spontaneously in broth from a "life force." (c) Lazzaro Spallanzani, whose experiments with broth aimed to disprove those of Needham.

## 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.

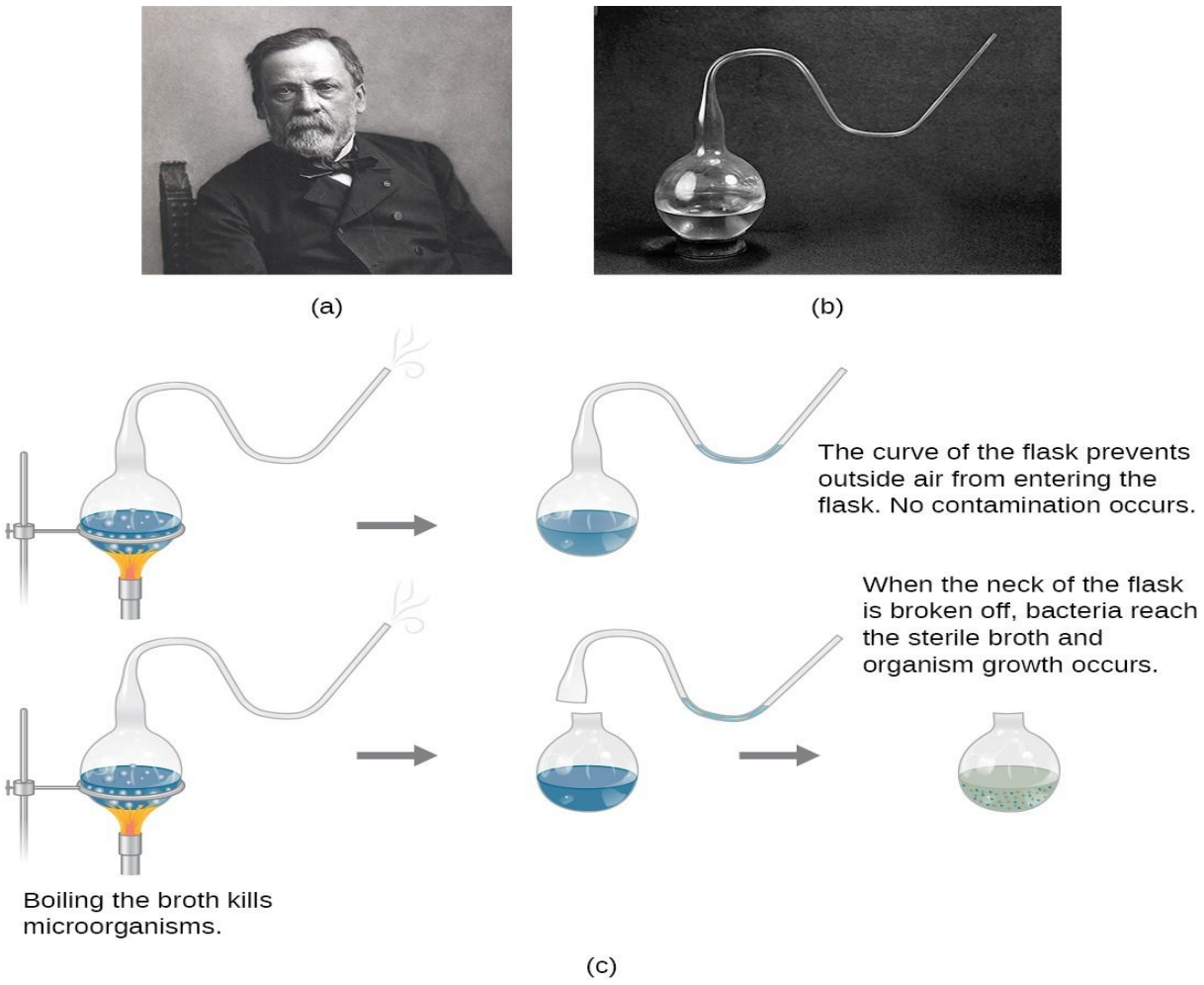


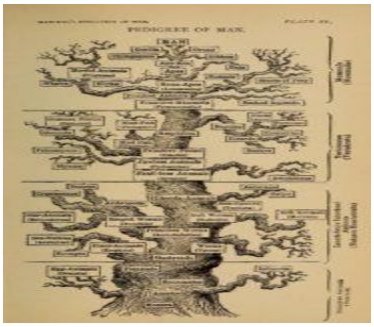

Figure 3. (a) French scientist Louis Pasteur, who definitively refuted the long-disputed theory of spontaneous generation. (b) The unique swan-neck feature of the flasks used in Pasteur's experiment allowed air to enter the flask but prevented the entry of bacterial and fungal spores. (c) Pasteur's experiment consisted of two parts. In the first part, the broth in the flask was boiled to sterilize it. When this broth was cooled, it remained free of contamination. In the second part of the experiment, the flask was boiled and then the neck was broken off. The broth in this flask became contaminated. (credit b: modification of work by "Wellcome Images"/Wikimedia Commons)

## The Theory of Biogenesis

### What is Biogenesis?

An important theory in biology and molecular genetics, Biogenesis postulates the production of new living organisms from pre-existing life. Read ahead as we explore this seminal theory that changed age-old beliefs.

Biogenesis is based on the theory that life can only come from life, and it refers to any process by which a lifeform can give rise to other life forms. For instance, a chicken laying eggs, which hatch and become baby chicken.




### Meaning of Biogenesis

This idea, however, contradicts the age-old hypothesis of spontaneous generation, i.e. some inorganic substances, if left alone for a few days, can give rise to life ( such as bacteria, vermin, maggots, etc.) as well.

The term 'biogenesis' comes from *'bio'* meaning 'life', and 'genesis', meaning 'beginning'. Rudolf Virchow, in 1858, had come up with the hypothesis of biogenesis, but could not experimentally prove it. In 1859, Louis Pasteur set up his demonstrative experiments to prove biogenesis right down to a bacterial level. By 1861, he succeeded in establishing biogenesis as a solid theory rather than a controversial hypothesis.

### What Was the Idea of Spontaneous Generation?

The belief in a spontaneous generation is age-old, quite literally. Aristotle in Ancient Greece first pronounces the idea. And consequently, the idea also came to be known as Aristotelian Abiogenesis.

The reason behind the resounding faith in this idea was perhaps the elusive and stealthy nature of the creatures attributed to it, i.e, mice, bacteria, flies, maggots, etc.

The 18th-century path-breaking invention of the microscope that allows most of these creatures, so we can observe them under the microscope and de-mystify their origin. By the time Pasteur set about to do his work in the field, macroscopic biogenesis was already accepted by the scientific community at large. He only had to confirm microscopic biogenesis to prove the hypothesis beyond doubt.

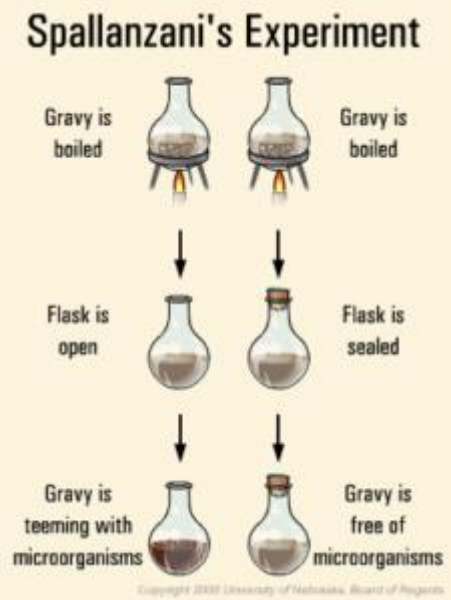
**Macroscopic Biogenesis: Francesco Redi's Experiment**

Francesco Redi, as far back as 1668, had set out to refute the idea of macroscopic spontaneous generation, by publishing the results of his experimentation on the matter. Instead of his experiment, Redi had placed some rotting meat in two containers, one with a piece of gauze covering the opening, and the other without it.


He noticed that in the container without the gauze, maggots would grow on the meat itself. However, when he provided the gauze, the maggots would appear on the gauze instead of on the meat. He also observed that flies tend to lay eggs as close to a food source as possible. Thus, he surmised the possibility of macroscopic biogenesis.

**Microscopic Biogenesis**

Spallanzani's Experiment



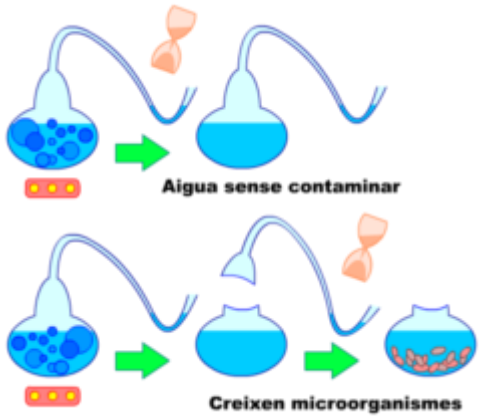

In 1768, Lazzaro Spallanzani suspected microscopic biogenesis and wanted to prove it experimentally. He boiled meat broth in a sealed container to avoid contamination. However, he was faced with a problem- upon heating a sealed container, the air inside would expand massively and would shatter the glass of the container.

He solved this problem by drawing out all the air in the container after sealing it. After experimenting with this manner, he achieved his desired results of a broth that had not clouded with bacterial growth, in line with the theory of biogenesis.

However, his inference was countered by critics who asserted that air was indispensable to support life, therefore the lack of bacterial growth should be attributed to the lack of air, rather than the fact that bacteria spread through contamination. For almost a century since this criticism lay unchallenged.

**Pasteur's Experiment**

The caveat of Pasteur's 1859 experiment was to establish that microbes live suspended in air, and can contaminate food and water, however, the microbes do not simply appear out of thin air. As the primary step to his experiment, Pasteur boiled beef broth in a special flask that had its long neck bent downwards and then upwards.




This interesting contraption ensured the free diffusion of air, and at the same time prevent any bacterial contamination. As long as the apparatus remained upright, the flask remained free of any bacterial growth.

Once we slant the flask, it allows the broth to pass beyond the 'goose-neck' bend of the flask's neck. The broth became clouded with bacterial growth in no time. This path-breaking experiment not only silenced all the criticism based on Spallanzani's experiment but also cemented the Law of Biogenesis.

**Law of Biogenesis Vs. Evolutionary Theory**

Scientist fears that the law of biogenesis opposes the theory of evolution. It has surmised that all life stems from inorganic matter from billions of years ago. However, biogenesis simply refutes the theory of spontaneous generation and delves in a matter of generational time-span, and not of what may be achieved over thousands of generations.

While the evolutionary theories take into account the lack of predators, the difference in the chemical composition of the Earth's atmosphere during the inception of life on Earth, as well as the trial-and-error that had taken place over millions of years to bring us to the stage of life on this planet we witness now, these do not concern the law of biogenesis at all.

Whereas the evolutionary theory demonstrates how life on earth took millions of years of trial-and-error and conducive but very different atmospheric conditions, the theory of spontaneous generation had asserted that complex life could simply appear fully formed in a matter of days. This is the belief that biogenesis had successfully challenged.

**Antonie van Leeuwenhoek**, (born October 24, 1632, Delft, Netherlands—died August 26, 1723, Delft), Dutch microscopist who was the first to observe bacteria and protozoa. His researches on lower animals refuted the doctrine of spontaneous generation, and his observations helped lay the foundations for the sciences of bacteriology and protozoology.

## Early Life And Career

At a young age, Leeuwenhoek lost his biological father. His mother later married painter Jacob Jansz Molijn. When his stepfather died in 1648, Leeuwenhoek was sent to Amsterdam to become an apprentice to a linen draper. Returning to Delft when he was 20, he established himself as a draper and haberdasher. He was married in 1654 to a draper's daughter. By the time of her death, in 1666, the couple had five children, only one of whom survived childhood. Leeuwenhoek remarried in 1671; his second wife died in 1694.

In 1660 Leeuwenhoek obtained a position as chamberlain to the sheriffs of Delft. His income was thus secure, and it was thereafter that he began to devote much of his time to his hobby of grinding lenses and using them to study tiny objects.

## Discovery Of Microscopic Life

Leeuwenhoek made microscopes consisting of a single high-quality lens of very short focal length; at the time, such simple microscopes were preferable to the compound microscope, which increased the problem of chromatic aberration. Although Leeuwenhoek's studies lacked the organization of formal scientific research, his powers of careful observation enabled him to make discoveries of fundamental importance. In 1674 he likely observed protozoa for the first time and several years later bacteria. Those "very little animalcules" he was able to isolate from different sources, such as rainwater, pond and well water, and the human mouth and intestine. He also calculated their sizes.

In 1677 he described for the first time the spermatozoa from insects, dogs, and man, though Stephen Hamm probably was a codiscoverer. Leeuwenhoek studied the structure of the optic lens, striations in muscles, the mouthparts of insects, and the fine structure of plants and discovered parthenogenesis in aphids. In 1680 he noticed that yeasts consist of minute globular particles. He extended Marcello Malpighi's demonstration in 1660 of the blood capillaries by giving the first accurate description of red blood cells. In his observations on rotifers in 1702, Leeuwenhoek remarked that *in all falling rain, carried from gutters into water-butts, animalcules are to be found; and that in all kinds of water, standing in the open air, animalcules can turn up. For these animalcules can be carried over by the wind, along with the bits of dust floating in the air.*

## The Royal Society And Later Discoveries

A friend of Leeuwenhoek put him in touch with the Royal Society of England, to which he communicated by means of informal letters from 1673 until 1723 most of his discoveries and to which he was elected a fellow in 1680. His discoveries were for the most part made public in the society's *Philosophical Transactions*. The first representation of bacteria is to be found in a drawing by Leeuwenhoek in that publication in 1683.

His researches on the life histories of various low forms of animal life were in opposition to the doctrine that they could be produced spontaneously or bred from corruption. Thus, he showed that the weevils of granaries (in his time commonly supposed to be bred from wheat as well as in it) are really grubs hatched from eggs deposited by winged insects. His letter on the flea, in which he not only described its structure but traced out the whole history of its metamorphosis, is of great interest, not so much for the exactness of his observations as for an illustration of his opposition to the spontaneous generation of many lower organisms, such as "this minute and despised creature." Some theorists asserted that the flea was produced from sand, others from dust or the like, but Leeuwenhoek proved that it bred in the regular way of winged insects.

Leeuwenhoek carefully studied the history of the ant and was the first to show that what had been commonly reputed to be ants' eggs were really their pupae, containing the perfect insect nearly ready for emergence, and that the true eggs were much smaller and gave origin to maggots, or larvae. He argued that the sea mussel and other shellfish were not generated out of sand found at the seashore or mud in the beds of rivers at low water but from spawn, by the regular course of generation. He maintained the same to be true of the freshwater mussel, whose embryos he examined so carefully that he was able to observe how they were consumed by "animalcules," many of which, according to his description, must have included ciliates in conjugation, flagellates, and the *Vorticella*. Similarly, he investigated the generation of eels, which were at that time supposed to be produced from dew without the ordinary process of generation. The dramatic nature of his discoveries made him famous, and he was visited by many notables—including Peter I (the Great) of Russia, James II of England, and Frederick II (the Great) of Prussia.

## Methods Of Microscopy

Leeuwenhoek's methods of microscopy, which he kept secret, remain something of a mystery. During his lifetime he ground more than 500 lenses, most of which were very small—some no larger than a pinhead—and usually mounted them between two thin brass plates, riveted together. A large sample of those lenses, bequeathed to the Royal Society, were found to have magnifying powers in the range of 50 to, at the most, 300 times. In order to observe phenomena as small as bacteria, Leeuwenhoek must have employed some form of oblique illumination, or other technique, for enhancing the effectiveness of the lens, but this method he would not reveal. Leeuwenhoek continued his work almost to the end of his long life of 90 years.

## Contributions To Scientific Literature

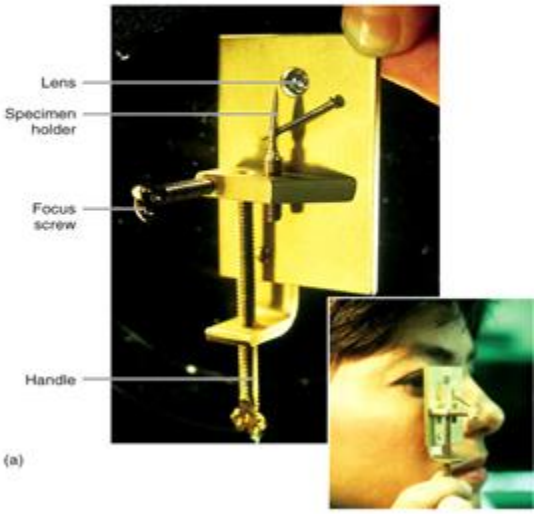
Leeuwenhoek's contributions to the *Philosophical Transactions* amounted to 375 and those to the *Memoirs of the Paris Academy of Sciences* to 27. Two collections of his works appeared during his life, one in Dutch (1685–1718) and the other in Latin (1715–22); a selection was translated by Samuel Hoole, *The Select Works of A. van Leeuwenhoek* (1798–18

## Antonie van Leeuwenhoek

- **Leeuwenhoek** (1632 –1723) was a Dutch tradesman and scientist.
- He is commonly known as "the Father of Microbiology", and considered to be the first microbiologist.
- He is best known for his work on the improvement of the microscope and for his contributions towards the establishment of microbiology.
- Using his handcrafted microscopes, he was the first to observe and describe single-celled organisms.

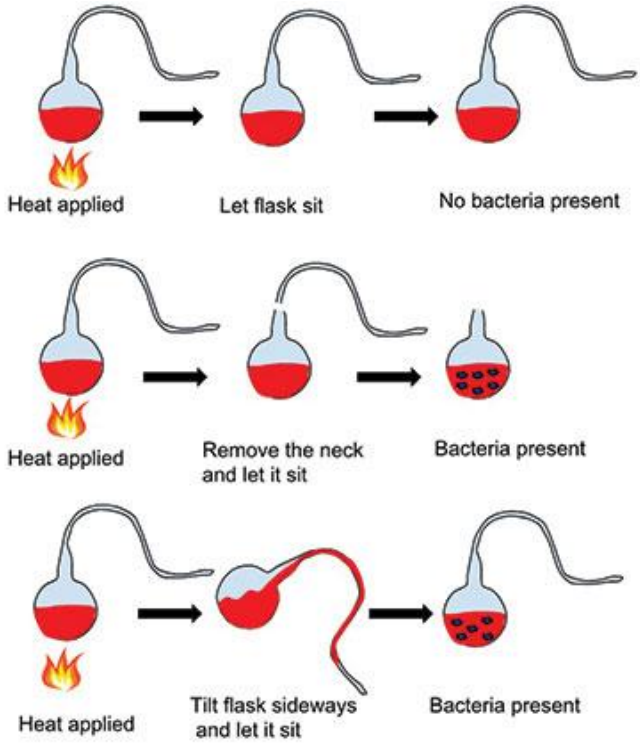
## Antonie van Leeuwenhoek

- In 1676, first to observe living microbes.
- His single-lens magnified up to 300X



## ***Louis Pasteur and his contributions***

- Louis Pasteur was a French chemist and microbiologist considered the most important founders of Microbiology.
- Microbiology developed as a scientific discipline from the era of Louis Pasteur (1822-1895) himself.
- He first coined the term "microbiology" for the study of organisms of microscopic size. For his innumerable contributions in the field, he is also known as the **Father of Microbiology**.
- He is renowned for his discoveries of the principles of **vaccination**, **microbial fermentation and pasteurization.**
- He is remembered for his remarkable breakthroughs in the causes and prevention of diseases.
- He is regarded as one of the three main founders of bacteriology, together with Ferdinand Cohn and Robert Koch.
- Pasteur's academic positions were numerous, and his scientific accomplishments earned him France's highest decoration, the Legion of Honor, as well as election to the Académie des Sciences and many other distinctions.
- Today there are some 30 institutes and an impressive number of hospitals, schools, buildings, and streets that bear his name- a set of honors bestowed on few scientists.




### Major Contributions of Louis Pasteur

The studies on fermentation led Pasteur to take interest to work in microbiology. His contributions to microbiology are as follows:

- He disproved the theory of spontaneous generation of disease and postulated the germ theory of disease: He stated that disease cannot be caused by bad air or vapor but it is produced by the microorganisms present in air.

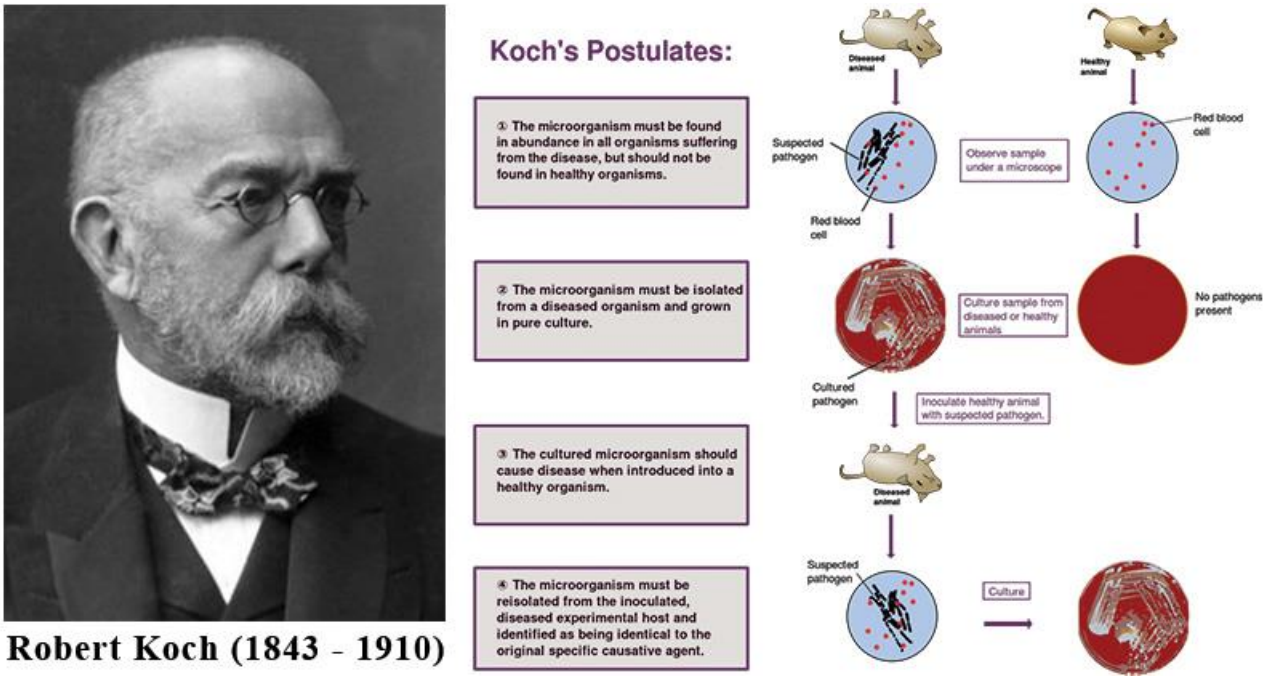
- The doctrine of spontaneous generation was disapproved by his experiments that showed that without contamination, microorganisms could not develop.
- He proposed the principles of fermentation for preservation of food.
- He introduced the sterilization techniques and developed steam sterilizer, hot air oven and autoclave.
- He described the method of pasteurization of milk and wine.
- He reduced mortality from puerperal fever. He had also contributed for the vaccine development against several diseases, such as anthrax, fowl cholera and rabies.
- Liquid media concept: He used nutrient broth to grow microorganisms.
- He was the founder of the Pasteur Institute, Paris.

Besides in microbiology, Pasteur made significant discoveries in chemistry, most notably on the molecular basis for the asymmetry of certain crystals and racemization.

- Early in his career, his investigation of tartaric acid resulted in the first resolution of what is now called optical isomers.
- His work led the way to the current understanding of a fundamental principle in the structure of organic compounds.

## ***Robert Koch and Koch's Postulates***

- **Heinrich Hermann Robert Koch** (1843 – 1910) provided remarkable contributions to the field of microbiology. He was a German general practitioner and a famous microbiologist.
- He is credited to be one of the founders of the specific field of modern bacteriology.
- As the founder, he identified the specific causative agents of tuberculosis, cholera, and anthrax and gave experimental support for the concept of infectious disease, which included experiments on humans and animals.
- For this he is also regarded as a pioneer of public health, aiding legislation and changing prevailing attitudes about hygiene to prevent the spread of various infectious diseases.
- For his work on tuberculosis, he was awarded the Nobel Prize in 1905 in Physiology or Medicine.




## Major Contributions of Robert Koch

- He investigated the anthrax disease cycle in 1876, and studied the bacteria that cause tuberculosis in 1882 and cholera in 1883.
- He discovered bacteria such as the anthrax bacilli, tubercle bacilli and cholera bacilli.
- Koch observed the phenomenon of acquired immunity.
- He introduced solid media for culture of bacteria. Koch pioneered the use of agar as a base for culture media. He developed the pour plate method and was the first to use solid culture media for culture of bacteria.
- Koch also developed media suitable for growing bacteria isolated from the body. Because of their similarity to body fluids, meat extracts and protein digests were used as nutrient sources. The result was the development of nutrient broth and nutrient agar media that are still in wide use today.
- He also introduced methods for isolation of bacteria in pure culture.
- He described hanging drop method for testing motility.
- He introduced staining techniques by using aniline dye.
- He invented the hot air oven and steam sterilizer, and also introduced methods to find out the efficacy of antiseptics.
- **Koch's Phenomenon:** Robert Koch observed that guinea pigs already infected with tubercle bacilli developed a hypersensitivity reaction when injected with tubercle bacilli or its protein. Since then, this observation was called as Koch's phenomenon.
- The medical applications of biotechnology still heavily depend on the Koch's principles of affirming the causes of infectious diseases.

## Contribution to the Germ theory

- Building on the early work of **Louis Pasteur** and the germ theory of disease, Robert Koch established the basic scientific requirements used to demonstrate that each specific disease is caused by a specific microorganism.

- The first direct demonstration of the role of bacteria in causing disease came from the study of anthrax by the German physician.
- These requirements were based on Koch's experiments with anthrax isolated from diseased hosts, and are known as "Koch's Postulates".

## The Experiment

In the experiment, Koch injected healthy mice with a material from diseased animals, and the mice became ill. After transferring anthrax by inoculation through a series of 20 mice, he incubated a piece of spleen containing the anthrax bacillus in beef serum. The bacilli grew, reproduced, and produced spores. When the isolated bacilli or spores were injected into mice, anthrax developed.

During Koch's studies on bacterial diseases, it became necessary to isolate suspected bacterial pathogens. His criteria for proving the causal relationship between a microorganism and a specific disease are known as Koch's postulates.

## Koch's Postulates

Koch's Postulates consist of the following four rules:

1. The microorganism must be identified in all individuals affected by the disease, but not in healthy individuals.
2. The microorganism can be isolated from the diseased individual and grown in culture.
3. When introduced into a healthy individual, the cultured microorganism should cause disease.
4. The microorganism must then be re-isolated from the experimental host, and found to be identical to the original microorganism.

## Limitations of Koch's Postulates

While Koch's Postulates were developed as general guidelines for the identification of infectious causes of disease, there are some inherent limitations that could not be resolved at the time.

- Viruses were not yet able to be cultured during the 1800's. Thus, while it appeared that an infectious agent was responsible for certain diseases, the lack of available techniques to isolate and culture viruses meant that not all Koch's Postulates could be met.
- The third postulate stipulates that the experimental host "should" exhibit disease, not "must". This is because asymptomatic carriers, immunity, and genetic resistance are possible.
- Koch's Postulates do not account for prion diseases and other agents that cannot be grown in culture.
- Most of the human bacterial pathogens satisfy Koch's postulates except for those of *Mycobacterium leprae* and *Treponema pallidum*, the causative agent of leprosy and syphilis, respectively. Both these bacteria are yet to be grown in cell-free culture media.

Therefore, Koch's Postulates have subsequently been revised to account for recent molecular advances and are no longer an absolute requirement of infectious causality.

## Edward Jenner

**Edward Jenner**, (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.[2][3] 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.[4]

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".[5][6][7] 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.[5]

### Early life

Jenner's handwritten draft of the first vaccination is held at the Royal College of Surgeons in London

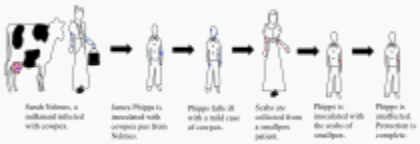
Edward Jenner was born on 17 May 1749[8] (6 May Old Style) in Berkeley, Gloucestershire, as the eighth of nine children. His father, the Reverend Stephen Jenner, was the vicar of Berkeley, so Jenner received a strong basic education.[8]

### Invention of the vaccine

*Edward Jenner advising a farmer to vaccinate his family*. Oil painting by an English painter, c. 1910 Jenner's discovery of the link between cowpox pus and smallpox in humans helped him to create the smallpox vaccine.

Inoculation was already a standard practice but involved serious risks, one of which was the fear that those inoculated would then transfer the disease to those around them due to their becoming carriers of the disease.[23] In 1721, Lady Mary Wortley Montagu had imported variolation to Britain after having observed it in Constantinople. While Johnnie Notions had great success with his self-devised inoculation[24] (and was reputed not to have lost a single patient),[25] his method's practice was limited to the Shetland Isles. Voltaire wrote that at this time 60% of the population caught smallpox and 20% of the population died of it.[26] Voltaire also states that

the Circassians used the inoculation from times immemorial, and the custom may have been borrowed by the Turks from the Circassians.[27]



The steps taken by Edward Jenner to create vaccination, the first vaccine for smallpox. Jenner did this by inoculating James Phipps with cowpox, a virus similar to smallpox, to create immunity, unlike variolation, which used smallpox to create an immunity to itself.

By 1768, English physician John Fewster had realised that prior infection with cowpox rendered a person immune to smallpox.[28] In the years following 1770, at least five investigators in England and Germany (Sevel, Jensen, Jesty 1774, Rendell, Plett 1791) successfully tested in humans a cowpox vaccine against smallpox.[29] For example, Dorset farmer Benjamin Jesty[30] successfully vaccinated and presumably induced immunity with cowpox in his wife and two children during a smallpox epidemic in 1774, but it was not until Jenner's work that the procedure became widely understood. Jenner may have been aware of Jesty's procedures and success.[31] A similar observation was later made in France by Jacques Antoine Rabaut-Pommier in 1780.[32]

Noting the common observation that milkmaids were generally immune to smallpox, Jenner postulated that the pus in the blisters that milkmaids received from cowpox (a disease similar to smallpox, but much less virulent) protected them from smallpox.

**Jenner's Hypothesis:**

The initial source of infection was a disease of horses, called "the grease", which was transferred to cattle by farm workers, transformed, and then manifested as cowpox.

Dr Jenner performing his first vaccination on James Phipps, a boy of age 8. 14 May 1796

On 14 May 1796, Jenner tested his hypothesis by inoculating James Phipps, an eight-year-old boy who was the son of Jenner's gardener. He scraped pus from cowpox blisters on the hands of Sarah Nelmes, a milkmaid who had caught cowpox from a cow called Blossom,[33] whose hide now hangs on the wall of the St. George's Medical School library (now in Tooting). Phipps was the 17th case described in Jenner's first paper on vaccination.[34]

Jenner inoculated Phipps in both arms that day, subsequently producing in Phipps a fever and some uneasiness, but no full-blown infection. Later, he injected Phipps with variolous material, the routine method of immunization at that time. No disease followed. The boy was later challenged with variolous material and again showed no sign of infection.

| |
|---|
| **Known:** |
| Smallpox is more dangerous than variolation and cowpox less dangerous than variolation. |
| **Hypothesis:** |
| If target is infected with cowpox, then target is immune to smallpox. |
| **Test:** |
| If variolation after infection with cowpox fails to produce a smallpox infection, immunity to smallpox has been achieved. |
| **Consequence:** |
| Immunity to smallpox can be induced much more safely than by variolation. |

## Death

Jenner was found in a state of apoplexy on 25 January 1823, with his right side paralysed. He did not recover and died the next day of an apparent stroke, his second, on 26 January 1823, aged 73. He was buried in the family vault at the Church of St Mary, Berkeley.[45] He was survived by his son Robert Fitzharding (1797–1854) and his daughter Catherine (1794–1833), his elder son Edward (1789–1810) having died of tuberculosis at age 21.[46]

## Paul Ehrlich

**Paul Ehrlich** (German: 14 March 1854 – 20 August 1915) was a Nobel prize-winning German physician and scientist who worked in the fields of hematology, immunology, and antimicrobial chemotherapy. Among his foremost achievements were finding a cure for syphilis in 1909, and inventing the precursor technique to Gram staining bacteria. The methods he developed for staining tissue made it possible to distinguish between different types of blood cells, which led to the ability to diagnose numerous blood diseases.

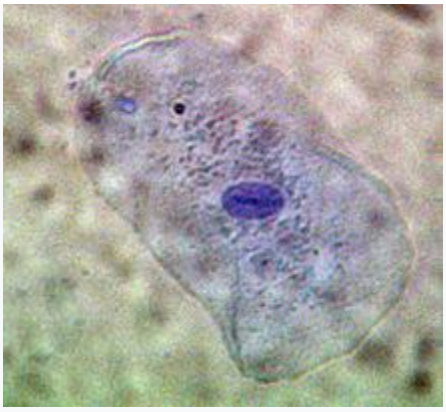
His laboratory discovered arsphenamine (Salvarsan), the first effective medicinal treatment for syphilis, thereby initiating and also naming the concept of chemotherapy. Ehrlich popularized the concept of a magic bullet.

### Chemotherapy

#### *In vivo staining*

In 1885 Ehrlich's monograph "The Need of the Organism for Oxygen," (*Das Sauerstoffbedürfnis des Organismus- Eine farbenanalytische Studie*) appeared, which he also submitted as a habilitation thesis. In it he introduced the new technology of in vivo staining. One of his findings was that pigments can only be easily assimilated by living organisms if they are in granular form. He injected the dyes alizarin blue and indophenol blue into laboratory animals and established after their death that various organs had been colored to different degrees. In organs with high oxygen saturation, indophenol was retained; in organs with medium saturation, indophenol was reduced, but not alizarin blue. And in areas with low oxygen saturation, both pigments were reduced. With this work, Ehrlich also formulated the conviction which guided his research: that all life processes can be traced to processes of physical chemistry occurring in the cell.

#### *Methylene blue*



Staining *in vivo* with methylene blue of a cell from the mucous membrane of a human mouth

In the course of his investigations Ehrlich came across methylene blue, which he regarded as particularly suitable for staining bacteria. Later, Robert Koch also used methylene blue as a dye in his research on the tuberculosis pathogen. In Ehrlich's view, an added benefit was that methylene blue also stained the long appendages of nerve cells, the axons. He initiated a doctoral

dissertation on the subject, but did not follow up the topic himself. It was the opinion of the neurologist Ludwig Edinger that Ehrlich had thereby opened up a major new topic in the field of neurology.

After mid-1889, when Ehrlich was unemployed, he privately continued his research on methylene blue. His work on in vivo staining gave him the idea of using it therapeutically. Since the parasite family of *Plasmodiidae* – which includes the malaria pathogen – can be stained with methylene blue, he thought it could possibly be used in the treatment of malaria. In the case of two patients so treated at the city hospital in Berlin-Moabit, their fever indeed subsided and the malaria plasmodia disappeared from their blood. Ehrlich obtained methylene blue from the company Meister Lucius & Brüning AG (later renamed Hoechst AG), which started a long collaboration with this company.

### *The search for a* chemotherapia specifica

Before the Institute of Experimental Therapy had moved to Frankfurt, Ehrlich had already resumed work on methylene blue. After the death of Georg Speyer, his widow Franziska Speyer endowed the Georg-Speyer House in his memory[13] which was erected next door to Ehrlich's institute. As director of the Georg-Speyer House, Ehrlich transferred his chemotherapeutic research there. He was looking for an agent which was as effective as methylene blue, but without its side effects. His model was on the one hand the impact of quinine on malaria, and on the other hand, in analogy to serum therapy, he thought there must also be chemical pharmaceuticals which would have just as specific an effect on individual diseases. His goal was to find a "Therapia sterilisans magna," in other words a treatment that could kill all disease pathogens.

Ehrlich and Sahachiro Hata

As a model for experimental therapy Ehrlich used a guinea pig disease trypanosoma and tested out various chemical substances on laboratory animals. The trypanosomes could indeed be successfully killed with the dye trypan red. Beginning in 1906, he intensively investigated atoxyl and had it tested by Robert Koch along with other arsenic compounds during Koch's sleeping sickness expedition of 1906/07. Although the name literally means “nonpoisonous,” atoxyl does cause damage, especially to the optic nerve. Ehrlich elaborated the systematic testing of chemical compounds in the sense of screening as now practiced in the

pharmaceutical industry. He discovered that Compound 418 - Arsenophenylglycine - had an impressive therapeutic effect and had it tested in Africa.

With the support of his assistant Sahachiro Hata Ehrlich discovered in 1909 that Compound 606, Arsphenamine, effectively combatted "spirillum" spirochaetes bacteria, one of whose subspecies causes syphilis.[14] The compound proved to have few side effects in human trials, and the spirochetes disappeared in seven syphilis patients after this treatment.

After extensive clinical testing (all the research participants had the negative example of tuberculin in mind) the Hoechst company began to market the compound toward the end of 1910 under the name Salvarsan. This was the first agent with a specific therapeutic effect to be created on the basis of theoretical considerations. Salvarsan proved to be amazingly effective, particularly when compared with the conventional therapy of mercury salts. Manufactured by Hoechst AG, Salvarsan became the most widely prescribed drug in the world. It was the most effective drug for treating syphilis until penicillin became available in the 1940s.[15] Salvarsan required improvement as to side effects and solubility and was replaced in 1911 with Neosalvarsan. Ehrlich's work illuminated the existence of the blood-brain barrier, although he himself never believed in such a barrier, with Lina Stern later coining the phrase.

The medication triggered the so-called "Salvarsan war." On one side there was hostility on the part of those who feared a resulting moral breakdown of sexual inhibitions. Ehrlich was also accused, with clearly anti-Semitic undertones, of excessively enriching himself. In addition, Ehrlich's associate, Paul Uhlenhuth claimed priority in discovering the drug.

Because some people died during the clinical testing, Ehrlich was accused of "stopping at nothing." In 1914, one of the most prominent accusers was convicted of criminal libel at a trial for which Ehrlich was called to testify. Though Ehrlich was thereby exonerated, the ordeal threw him into a depression from which he never fully recovered.[16]

**Magic bullet**

Ehrlich reasoned that if a compound could be made that selectively targeted a disease-causing organism, then a toxin for that organism could be delivered along with the agent of selectivity. Hence, a "magic bullet" (*Zauberkugel*, his term for an ideal therapeutic agent) would be created that killed only the organism targeted. The concept of a "magic bullet" has to some extent been realized by the development of antibody-drug conjugates (a monoclonal antibody linked to a cytotoxic biologically active drug), as they enable cytotoxic drugs to be selectively delivered to their designated targets (e.g. cancer cells).

**Honors and titles**[edit]

- 1882 Awarded the title of Professor
- 1890 Appointed Extraordinary Professor at the *Friedrich-Wilhelms-Universität* (now Humboldt University)
- 1896 Given the nonacademic Prussian title of a Medical Councillor (*Geheimer Medizinalrat*)
- 1903 Awarded Prussia's highest distinction in science, the Great Golden Medal of Science (which had previously been awarded only to Rudolf Virchow)
- 1904 Honorary professorship in Göttingen;[19] honorary doctorate from the University of Chicago

- 1907 Granted the seldom-awarded title Senior Medical Councillor (*Geheimer Obermedizinalrat*); granted an honorary doctorate from Oxford University
- 1908 Awarded The Nobel Prize in Physiology or Medicine for his “work on immunity"[20][21]
- 1911 Granted Prussia's highest civilian award, Privy Councillor (*Wirklicher Geheimer Rat* with the predicate “Excellency”)
- 1912 Made an honorary citizen of the city of Frankfurt a.M. and of his birthplace Strehlen
- 1914 Awarded the Cameron Prize for Therapeutics of the University of Edinburgh
- 1914 Appointed full Professor of Pharmacology at the newly established Frankfurt University.

## Sir Alexander Fleming

**Sir Alexander Fleming** (6 August 1881 – 11 March 1955) was a Scottish physician and microbiologist, best known for discovering the enzyme lysozyme and the world's first broadly effective antibiotic substance which he named penicillin. He discovered lysozyme from his nasal discharge in 1922, and along with it a bacterium he named *Micrococcus Lysodeikticus*, later renamed *Micrococcus luteus*. His discovery of what is later named benzylpenicillin (or penicillin G) from the mould *Penicillium rubens* in 1928, is described as the "single greatest victory ever achieved over disease."[3][4] For this discovery he shared the Nobel Prize in Physiology or Medicine in 1945 with Howard Florey and Ernst Boris Chain.

Scientific contributions

### Antiseptics

During World War I, Fleming with Leonard Colebrook and Sir Almroth Wright joined the war efforts and practically moved the entire Inoculation Department of St Mary's to the British military hospital at Boulogne-sur-Mer. Serving as Temporary Lieutenant of the Royal Army Medical Corps, he witnessed the death of many soldiers from sepsis resulting from infected wounds. Antiseptics, which were used at the time to treat infected wounds, he observed, often worsened the injuries.[12] In an article published in the medical journal *The Lancet* in 1917, he described an ingenious experiment, which he was able to conduct as a result of his own glass blowing skills, in which he explained why antiseptics were killing more soldiers than infection itself during the war. Antiseptics worked well on the surface, but deep wounds tended to shelter anaerobic bacteria from the antiseptic agent, and antiseptics seemed to remove beneficial agents produced that protected the patients in these cases at least as well as they removed bacteria, and did nothing to remove the bacteria that were out of reach.[13] Wright strongly supported Fleming's findings, but despite this, most army physicians over the course of the war continued to use antiseptics even in cases where this worsened the condition of the patients.[9]

### Discovery of lysozyme

At St Mary's Hospital, Fleming continued his investigations into bacteria culture and antibacterial substances. As his research scholar at the time V.D. Allison recalled, Fleming was not a tidy researcher and usually expected unusual bacterial growths in his culture plates. Fleming had tease Allison of his "excessive tidiness in the laboratory," and Allison rightly

attributed such untidiness as the success of Fleming's experiments, and said, "[If] he had been as tidy as he thought I was, he would not have made his two great discoveries."

In the late 1921, while he was maintaining agar plates for bacteria, he found that one of the plates was contaminated with bacteria from the air. When he added nasal mucus, he found that the mucus inhibited the bacterial growth.[15] Surrounding the mucus area was a clear transparent circle (1 cm from the mucus), indicating the killing zone of bacteria, followed by a glassy and translucent ring beyond which was an opaque area indicating normal bacterial growth. In the next test, he used bacteria maintained in saline that formed an yellow suspension. Within two minutes of adding fresh mucus, the yellow saline turned completely clear. He extended his tests using tears, which were contributed by his co-workers. As Allison reminisced, saying, "For the next five or six weeks, our tears were the source of supply for this extraordinary phenomenon. Many were the lemons we used (after the failure of onions) to produce a flow of tears... The demand by us for tears was so great, that laboratory attendants were pressed into service, receiving threepence for each contribution."[14]

His further tests with sputum, cartilage, blood, semen, ovarian cyst fluid, pus, and egg white showed that the bactericidal agent was present in all of these.[16] He reported his discovery before the Medical Research Club in December and before the Royal Society the next year but failed to stir any interest, as Allison recollected:

I was present at this [Medical Research Club] meeting as Fleming's guest. His paper describing his discovery was received with no questions asked and no discussion, which was most unusual and an indication that it was considered to be of no importance. The following year he read a paper on the subject before the Royal Society, Burlington House, Piccadilly and he and I gave a demonstration of our work. Again with one exception little comment or attention was paid to it.[14]

Reporting in the 1 May 1922 issue of the *Proceedings of the Royal Society B: Biological Sciences* under the title "On a remarkable bacteriolytic element found in tissues and secretions," Fleming wrote:

In this communication I wish to draw attention to a substance present in the tissues and secretions of the body, which is capable of rapidly dissolving certain bacteria. As this substance has properties akin to those of ferments I have called it a "Lysozyme," and shall refer to it by this name throughout the communication. The lysozyme was first noticed during some investigations made on a patient suffering from acute coryza.[15]

This was the first recorded discovery of lysozyme. With Allison, he published further studies on lysozyme in October issue of the *British Journal of Experimental Pathology* the same year.[17] Although he was able to obtain larger amounts of lysozyme from egg whites, the enzyme was only effective against small counts of harmless bacteria, and therefore had little therapeutic potential. This indicates one of the major differences between pathogenic and harmless bacteria.[12] Described in the original publication, "a patient suffering from acute coryza"[15] was later identified as Fleming himself. His research notebook dated 21 November 1921 showed a sketch of the culture plate with a small note: “Staphyloid coccus from A.F.'s nose."[16] He also identified the bacterium present in the nasal mucus as *Micrococcus Lysodeikticus*, giving the species name (meaning "lysis indicator" for its susceptibility to lysozymal activity).[18] The species was reassigned as *Micrococcus luteus* in 1972.[19] The "Fleming strain" (NCTC2665) of this bacterium has become a model in different biological

studies.[20][21] The importance of lysozyme was not recognised, and Fleming was well aware of this, in his Presidential address at the Royal Society of Medicine meeting on 18 October 1932, he said:

I choose lysozyme as the subject for this address for two reasons, firstly because I have a fatherly interest in the name, and, secondly, because its importance in connection with natural immunity does not seem to be generally appreciated.[22]

In his Nobel lecture on 11 December 1945 he briefly mentioned lysozyme, saying, "Penicillin was not the first antibiotic I happened to discover."[23] It was only towards the end of the 20th century that the true importance of Fleming's discovery in immunology was realised as lysozyme became the first antimicrobial protein discovered that constitute part of our innate immunity.[24][25]

**Discovery of penicillin**


An advertisement advertising penicillin's "miracle cure".

One sometimes finds, what one is not looking for. When I woke up just after dawn on September 28, 1928, I certainly didn't plan to revolutionize all medicine by discovering the world's first antibiotic, or bacteria killer. But I suppose that was exactly what I did.

— *Alexander Fleming*[26]

***Experiment***

By 1927, Fleming had been investigating the properties of staphylococci. He was already well known from his earlier work, and had developed a reputation as a brilliant researcher. In 1928, he studied the variation of *Staphylococcus aureus* grown under natural condition, after the work of Joseph Warwick Bigger, who discovered that the bacterium could grow into a variety of types (strains).[27] On 3 September 1928, Fleming returned to his laboratory having spent a holiday with his family at Suffolk. Before leaving for his holiday, he inoculated staphylococci on culture plates and left them on a bench in a corner of his laboratory.[16] On his return, Fleming noticed that one culture was contaminated with a fungus, and that the colonies of staphylococci immediately surrounding the fungus had been destroyed, whereas other staphylococci colonies farther away were normal, famously remarking "That's funny".[28] Fleming showed the contaminated culture to his former assistant Merlin Pryce, who reminded him, "That's how you discovered lysozyme."[29] He identified the mould as being from the genus *Penicillium.* He suspected it to be *P. chrysogenum*, but a colleague Charles J. La Touche identified it as *P.*

*rubrum*. (It was later corrected as *P. notatum* and then officially accepted as *P. chrysogenum*; but finally in 2011, it was resolved as *P. rubens*.)[30][31]

The laboratory in which Fleming discovered and tested penicillin is preserved as the Alexander Fleming Laboratory Museum in St. Mary's Hospital, Paddington. The source of the fungal contaminant was established in 1966 as coming from La Touche's room, which was directly below Fleming's.[32][33]

Fleming grew the mould in a pure culture and found that the culture broth contained an antibacterial substance. He investigated its anti-bacterial effect on many organisms, and noticed that it affected bacteria such as staphylococci and many other Gram-positive pathogens that cause scarlet fever, pneumonia, meningitis and diphtheria, but not typhoid fever or paratyphoid fever, which are caused by Gram-negative bacteria, for which he was seeking a cure at the time. It also affected *Neisseria gonorrhoeae*, which causes gonorrhoea, although this bacterium is Gram-negative. After some months of calling it "mould juice" or "the inhibitor", he gave the name penicillin on 7 March 1929 for the antibacterial substance present in the mould.[34]

***Reception and publication***

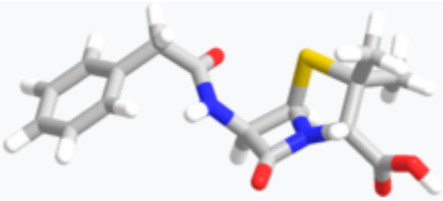
Fleming presented his discovery on 13 February 1929 before the Medical Research Club. His talk on "A medium for the isolation of Pfeiffer's bacillus" did not receive any particular attention or comment. Henry Dale, the then Director of National Institute for Medical Research and chair of the meeting, much later reminisced that he did not even sense any striking point of importance in Fleming's speech.[16] Fleming published his discovery in 1929 in the *British Journal of Experimental Pathology*,[35] but little attention was paid to the article. His problem was the difficulty of producing penicillin in large amounts, and moreover, isolation of the main compound. Even with the help of Harold Raistrick and his team of biochemists at the London School of Hygiene and Tropical Medicine, chemical purification was futile. "As a result, penicillin languished largely forgotten in the 1930s," as Milton Wainwright described.[36]

As late as in 1936, there was no appreciation for penicillin. When Fleming talked of its medical importance at the Second International Congress of Microbiology held in London,[37][38] no one believed him. As Allison, his companion in both the Medical Research Club and international congress meeting, remarked the two occasions:

[Fleming at the Medical Research Club meeting] suggested the possible value of penicillin for the treatment of infection in man. Again there was a total lack of interest and no discussion. Fleming was keenly disappointed, but worse was to follow. He read a paper on his work on penicillin at a meeting of the International Congress of Microbiology, attended by the foremost bacteriologists from all over the world. There was no support for his views on its possible future value for the prevention and treatment of human infections and discussion was minimal. Fleming bore these disappointments stoically, but they did not alter his views or deter him from continuing his investigation of penicillin.[14]

In 1941, the *British Medical Journal* reported that "[Penicillin] does not appear to have been considered as possibly useful from any other point of view."[39][40][32]

***Purification and stabilisation***



3D-model of benzylpenicillin

In Oxford, Ernst Boris Chain and Edward Abraham were studying the molecular structure of the antibiotic. Abraham was the first to propose the correct structure of penicillin.[41][42] Shortly after the team published its first results in 1940, Fleming telephoned Howard Florey, Chain's head of department, to say that he would be visiting within the next few days. When Chain heard that Fleming was coming, he remarked "Good God! I thought he was dead."[43]

Norman Heatley suggested transferring the active ingredient of penicillin back into water by changing its acidity. This produced enough of the drug to begin testing on animals. There were many more people involved in the Oxford team, and at one point the entire Sir William Dunn School of Pathology was involved in its production. After the team had developed a method of purifying penicillin to an effective first stable form in 1940, several clinical trials ensued, and their amazing success inspired the team to develop methods for mass production and mass distribution in 1945.[44][45]

Fleming was modest about his part in the development of penicillin, describing his fame as the "Fleming Myth" and he praised Florey and Chain for transforming the laboratory curiosity into a practical drug. Fleming was the first to discover the properties of the active substance, giving him the privilege of naming it: penicillin. He also kept, grew, and distributed the original mould for twelve years, and continued until 1940 to try to get help from any chemist who had enough skill to make penicillin. But Sir Henry Harris said in 1998: "Without Fleming, no Chain; without Chain, no Florey; without Florey, no Heatley; without Heatley, no penicillin."[46] The discovery of penicillin and its subsequent development as a prescription drug mark the start of modern antibiotics.[47]

***Medical use and mass production***

In his first clinical trial, Fleming treated his research scholar Stuart Craddock who had developed severe infection of the nasal antrum (sinusitis). The treatment started on 9 January 1929 but without any effect. It probably was due to the fact that the infection was with influenza bacillus (*Haemophilus influenzae*), the bacterium which he had found unsusceptible to penicillin.[32] Fleming gave some of his original penicillin samples to his colleague-surgeon Arthur Dickson Wright for clinical test in 1928.[48][49] Although Wright reportedly said that it "seemed to work satisfactorily,"[50] there are no records of its specific use. Cecil George Paine, a pathologist at the Royal Infirmary in Sheffield and former student of Fleming, was the first to use penicillin successfully for medical treatment.[36] He cured eye infections (conjunctivitis) of one adult and three infants (neonatal conjunctivitis) on 25 November 1930.[51]

Fleming also successfully treated severe conjunctivitis in 1932.[3][52][53] Keith Bernard Rogers, who had joined St Mary's as medical student in 1929,[54] was captain the London University rifle team and was about to participate in inter-hospital rifle shooting competition when he developed

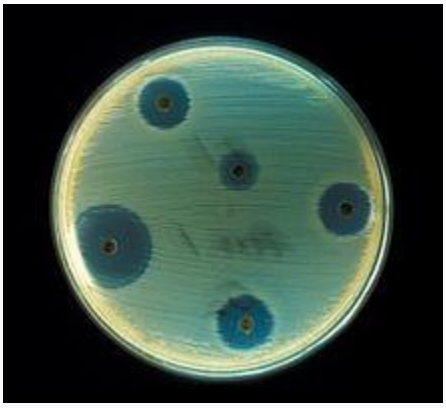
conjunctivitis.[55][56][57] Fleming applied his penicillin and cured Rogers before the competition.[3][52][58] It is said that the "penicillin worked and the match was won." However, the report that "Keith was probably the first patient to be treated clinically with penicillin ointment"[56] is no longer true as Paine's medical records showed up.[34]

There is a popular assertion both in popular and scientific literature that Fleming largely abandoned penicillin work in the early 1930s.[59][60][61][62][63][64][65][66] In his review of André Maurois's *The Life of Sir Alexander Fleming, Discoverer of Penicillin*, William L. Kissick went so far as to say that "Fleming had abandoned penicillin in 1932... Although the recipient of many honors and the author of much scientific work, Sir Alexander Fleming does not appear to be an ideal subject for a biography."[67] This is a false information, as Fleming continued to pursue penicillin research.[49][68] As late as in 1939, Fleming's notebook shows attempts to make better penicillin production using different media.[34] In 1941, he published a method for assessment of penicillin effectiveness.[69] As to the chemical isolation and purification, Howard Florey and Ernst Boris Chain at the Radcliffe Infirmary in Oxford took up the research research to mass-produce it, and achieved with supports from World War II military projects under the U.S. and British governments.[70]

By mid-1942, the Oxford team produced the pure penicillin compound as yellow powder.[71] In August 1942, Harry Lambert (an associate of Fleming's brother Robert) was admitted to St Mary's Hospital due to life-threatening infection of the nervous system (streptococcal meningitis).[72] Fleming treated him with sulphonamides, but Lambert's condition deteriorated. He tested the antibiotic susceptibility and found that his penicillin could kill the bacteria. He requested Florey for the isolated sample. When Florey sent the incompletely purified sample, which Fleming immediately administered into Lambert's spinal canal. Lambert showed signs of improvement the very next day,[14] and completely recovered within a week.[3][73] Fleming published the clinical case in *The Lancet* in 1943.[74]

Upon this medical breakthrough, Allison informed the British Ministry of Health of the importance of penicillin and the need for mass production. The War Cabinet was convinced of the usefulness upon which Sir Cecil Weir, Director General of Equipment, called for a meeting on the mode of action on 28 September 1942.[75][76] The Penicillin Committee was created on 5 April 1943. The committee consisted of Weir as Chairman, Fleming, Florey, Sir Percival Hartley, Allison and representatives from pharmaceutical companies as members. The main goals were to produce penicillin rapidly in large quantities with collaboration of American companies, and to supply the drug exclusively for Allied armed forces.[14] By D-Day in 1944, enough penicillin had been produced to treat all the wounded of the Allied troops.[77]

***Antibiotic resistance***



Modern antibiotics are tested using a method similar to Fleming's discovery.

Fleming also discovered very early that bacteria developed antibiotic resistance whenever too little penicillin was used or when it was used for too short a period. Almroth Wright had predicted antibiotic resistance even before it was noticed during experiments. Fleming cautioned about the use of penicillin in his many speeches around the world. On 26 June 1945, he made the following cautionary statements: "the microbes are educated to resist penicillin and a host of penicillin-fast organisms is bred out ... In such cases the thoughtless person playing with penicillin is morally responsible for the death of the man who finally succumbs to infection with the penicillin-resistant organism. I hope this evil can be averted."[78] He cautioned not to use penicillin unless there was a properly diagnosed reason for it to be used, and that if it were used, never to use too little, or for too short a period, since these are the circumstances under which bacterial resistance to antibiotics develops.[79]

It had been experimentally shown in 1942 that *S. aureu*s could developed penicillin resistance under prolonged exposure.[80] Elaborating the possibility of penicillin resistance in clinical conditions in his Nobel Lecture, Fleming said:

The time may come when penicillin can be bought by anyone in the shops. Then there is the danger that the ignorant man may easily underdose himself and by exposing his microbes to non-lethal quantities of the drug make them resistant.[23]

It was around that time that the first clinical case of penicillin resistance was reported.[81]

Personal life

Grave of Sir Alexander Fleming in the crypt of St Paul's Cathedral, London.

On 24 December 1915, Fleming married a trained nurse, Sarah Marion McElroy of Killala, County Mayo, Ireland. Their only child, Robert Fleming (1924–2015), became a general medical practitioner. After his first wife's death in 1949, Fleming married Amalia Koutsouri-Vourekas, a Greek colleague at St. Mary's, on 9 April 1953; she died in 1986.

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## SCHOOL OF BIO AND CHEMICAL ENGINEERING

## DEPARTMENT OF BIOTECHNOLOGY

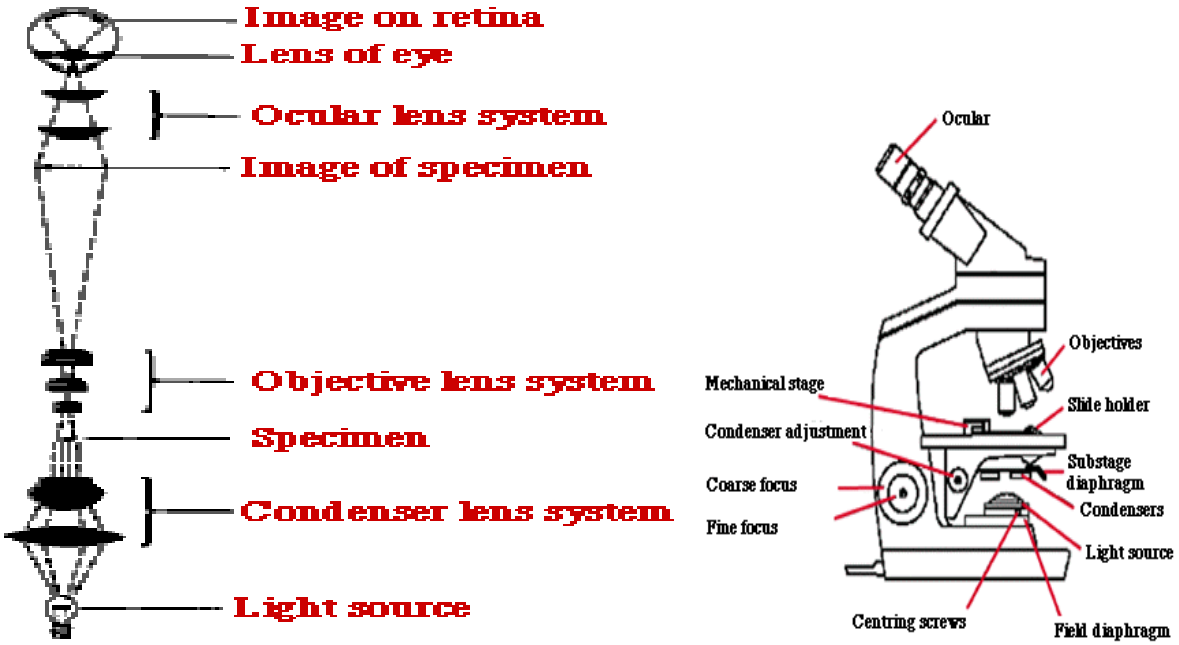
## B.SC. BIOTECHNOLOGY / BIOCHEMISTRY

## UNIT - 2 - FUNDAMENTALS OF MICROBIOLOGY - SBB1102

**COMPOUND / LIGHT MICROSCOPE**

1. **Bright field microscopy**

**Bright field microscopy** is the simplest of all the optical microscopy illumination techniques. Sample illumination is transmitted (i.e., illuminated from below and observed from above) white light and contrast in the sample is caused by absorbance of some of the transmitted light in dense areas of the sample. Bright field microscopy is the simplest of a range of techniques used for illumination of samples in light microscopes and its simplicity makes it a popular technique. The typical appearance of a bright field microscopy image is a dark sample on a bright background, hence the name.




**Light path**

The light path of a bright field microscope is extremely simple, no additional components are required beyond the normal light microscope setup. The light path therefore consists of:

**light source**

1. a transillumination light source, commonly a halogen lamp in the microscope stand; A **halogen lamp**, also known as a **tungsten halogen lamp** or **quartz iodine lamp**, is an incandescent lamp that has a small amount of a halogen such as iodine or bromine added. The combination of the halogen gas and the tungsten filament produces a **halogen cycle** chemical reaction which redeposits evaporated tungsten back onto the filament, increasing its life and maintaining the clarity of the envelope
2. a **condenser lens** which focuses light from the light source onto the sample. A **condenser** is one of the main components of the optical system of many transmitted light

compound microscopes. A condenser is a lens that serves to concentrate light from the illumination source that is in turn focused through the object and magnified by the objective lens.

3. **objective lens** : In an optical instrument, the **objective** is the optical element that gathers light from the object being observed and focuses the light rays to produce a real image. Objectives can be single lenses or mirrors, or combinations of several optical elements. Microscope objectives are characterized by two parameters: magnification and numerical aperture. The typically ranges are 4× , 10x , 40x and 100×.
4. **oculars** to view the sample image. An **eyepiece**, or **ocular lens**, is a type of lens that is attached to a variety of optical devices such as microscopes. It is so named because it is usually the lens that is closest to the eye when someone looks through the device. The objective lens or mirror collects light and brings it to focus creating an image. The eyepiece is placed near the focal point of the objective to magnify this image. The amount of magnification depends on the focal length of the eyepiece.

**Magnification** is the process of enlarging something only in appearance, not in physical size. Typically magnification is related to scaling up visuals or images to be able to see more detail, increasing resolution.

**Resolving power** is the ability of an imaging device to separate (i.e., to see as distinct) points of an object that are located at a small angular distance..

In optics, the **numerical aperture** (**NA**) of an optical system is a dimensionless number that characterizes the range of angles over which the system can accept or emit light. In most areas of optics, and especially in microscopy, the numerical aperture of an optical system such as an objective lens is defined by

$$\mathrm{NA} = n \sin\theta$$

where $n$ is the index of refraction of the medium in which the lens is working (1.0 for air, 1.33 for pure water, and up to 1.56 for oils; see also list of refractive indices), and $\theta$ is the half-angle of the maximum cone of light that can enter or exit the lens. In general, this is the angle of the real marginal ray in the system

**Working Performance**

Bright field microscopy typically has low contrast with most biological samples as few absorb light to a great extent. Staining is often required to increase contrast, which prevents use on live cells in many situations. Bright field illumination is useful for samples which have an intrinsic colour, for example chloroplasts in plant cells.

Light is first emitted by the light **source** and is directed by the **condenser lens** on to the specimen, which might be a loose object, a prepared plate or almost anything. A microscope can

even be applied to small parts of larger objects, though with a bit more difficulty. (The light does not absolutely need to originate below the specimen.)

The light from the specimen then passes through the objective lens. This lens is often selected from among three or four and is the main determinant for the level of magnification. It bends the light rays and in the case of this example sends them to a **projector lens**, which reverses their direction so that when the image reaches the eye it will not appear "upside-down". Not all microscopes have a projector lens, so the viewer may be seeing a reverse image. In these cases, when the slide is moved, it will appear to be moving in the opposite direction to the viewer.

The light rays then travel to the **oracular lens** or "eye piece". This is often a 10X magnification lens, meaning it magnifies the magnified image an additional ten times. The image is then projected into the eye. It is very seldom that a specimen is in focus the moment it is placed beneath a microscope. This means that some adjustment will have to be made. Unlike in telescopes, the focal length between lenses remains constant when adjusting the focus. The lens apparatus is brought closer to or further from the object. The focus adjustment is often along the neck of the tube containing the lenses, but it might just as well move the slide up and down. The best way to make this adjustment is to make a course adjustment so that it is too close to the object and then back off with the fine adjustment[2]. This helps to ensure that the specimen is not inadvertently smashed by the lens.

**Advantages**

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The name "brightfield" is derived from the fact that the specimen is dark and contrasted by the surrounding bright viewing field. Simple light microscopes are sometimes referred to as bright field microscopes.

Brightfield microscopy is very simple to use with fewer adjustments needed to be made to view specimens.

Some specimens can be viewed without staining and the optics used in the brightfield technique don’t alter the color of the specimen.

It is adaptable with new technology and optional pieces of equipment can be implemented with brightfield illumination to give versatility in the tasks it can perform.

**Disadvantages**

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Certain disadvantages are inherent in any optical imaging technique.

- By using an aperture diaphragm for contrast, past a certain point, greater contrast adds distortion. However, employing an iris diaphragm will help compensate for this problem.

- Brightfield microscopy can't be used to observe living specimens of bacteria, although when using fixed specimens, bacteria have an optimum viewing magnification of 1000x.

Brightfield microscopy has very low contrast and most cells absolutely have to be stained to be seen; staining may introduce extraneous details into the specimen that should not be present.

Also, the user will need to be knowledgeable in proper staining techniques.

Lastly, this method requires a strong light source for high magnification applications and intense lighting can produce heat that will damage specimens or kill living microorganisms.

2. **Dark field microscopy**

**Dark field microscopy** (dark ground microscopy) describes microscopy methods, in both light and electron microscopy, which exclude the unscattered beam from the image. As a result, the field around the specimen (i.e. where there is no specimen to scatter the beam) is generally dark.

In optical microscopy, darkfield describes an illumination technique used to enhance the contrast in unstained samples. It works by illuminating the sample with light that will not be collected by the objective lens, and thus will not form part of the image. This produces the classic appearance of a dark, almost black, background with bright objects on it.

**The light's path**

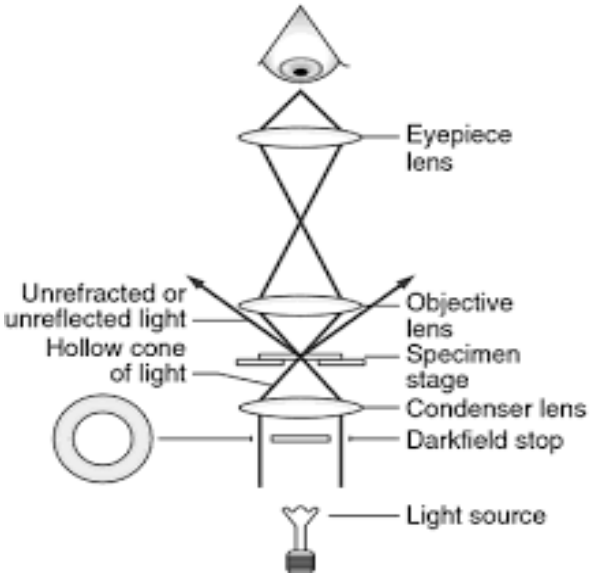



**Diagram illustrating the light path through a dark field microscope**.

1. Light enters the microscope for illumination of the sample.

2. A specially sized disc, the *patch stop* (see figure) blocks some light from the light source, leaving an outer ring of illumination. A wide phase annulus can also be reasonably substituted at low magnification.
3. The condenser lens focuses the light towards the sample.
4. The light enters the sample. Most is directly transmitted, while some is scattered from the sample.
5. The **scattered light** enters the objective lens, while the **directly transmitted light** simply misses the lens and is not collected due to a *direct illumination block* (see figure).
6. Only the scattered light goes on to produce the image, while the directly transmitted light is omitted.

Dark field microscopy produces an image with a dark background.

**Advantages and disadvantages**

Dark field microscopy is a very simple yet effective technique and well suited for uses involving live and unstained biological samples, such as a smear from a tissue culture or individual water-borne single-celled organisms. Considering the simplicity of the setup, the quality of images obtained from this technique is impressive.

The main limitation of dark field microscopy is the low light levels seen in the final image. This means the sample must be very strongly illuminated, which can cause damage to the sample. Dark field microscopy techniques are almost entirely free of artifacts, due to the nature of the process. However the interpretation of dark field images must be done with great care as common dark features of bright field microscopy images may be invisible, and vice versa.

While the dark field image may first appear to be a negative of the bright field image, different effects are visible in each. In bright field microscopy, features are visible where either a shadow is cast on the surface by the incident light, or a part of the surface is less reflective, possibly by the presence of pits or scratches. Raised features that are too smooth to cast shadows will not appear in bright field images, but the light that reflects off the sides of the feature will be visible in the dark field images.

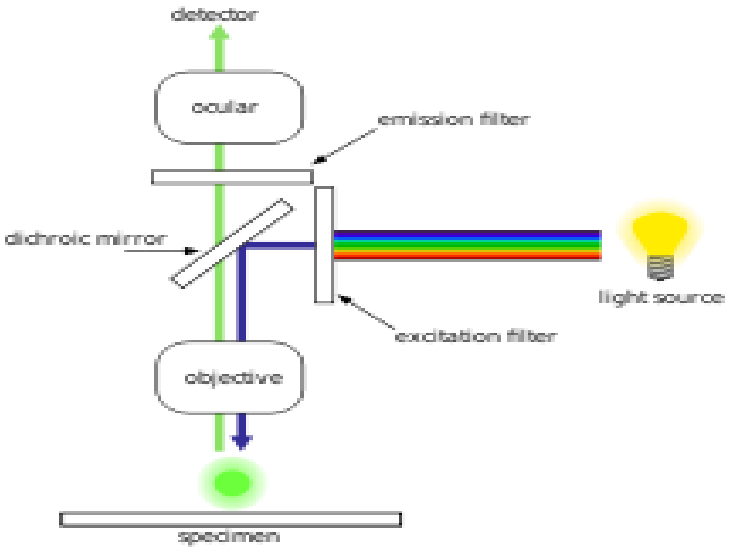
## 3. Fluorescence microscope

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.[1][2] 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.

### Principle

The specimen is illuminated with light of a specific wavelength (or wavelengths) which is absorbed by the fluorophores, causing them to emit light of longer wavelengths (i.e., of a different color than the absorbed light). The illumination light is separated from the much weaker emitted fluorescence through the use of a spectral emission filter. Typical components of a fluorescence microscope are a light source (xenon arc lamp or mercury-vapor lamp are common; more advanced forms are high-power LEDs and lasers), the excitation filter, the dichroic mirror (or dichroic beamsplitter), and the emission filter (see figure below). The filters and the dichroic are chosen to match the spectral excitation and emission characteristics of the fluorophore used to label the specimen.[1] In this manner, the distribution of a single fluorophore (color) is imaged at a time. Multi-color images of several types of fluorophores must be composed by combining several single-color images.[1]

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). These microscopes are widely used in biology and are the basis for more advanced microscope designs, such as the confocal microscope and the total internal reflection fluorescence microscope (TIRF).



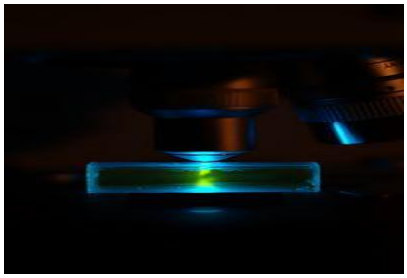

**Schematic of a fluorescence microscope**.

The majority of fluorescence microscopes, especially those used in the life sciences, are of the epifluorescence design shown in the diagram. Light of the excitation wavelength is focused on the specimen through the objective lens. The fluorescence emitted by the specimen is focused to the detector by the same objective that is used for the excitation which for greatest sensitivity will have a very high numerical aperture. Since most of the excitation light is transmitted through the specimen, only reflected excitatory light reaches the objective together with the emitted light and the epifluorescence method therefore gives a high signal to noise ratio. An additional barrier filter between the objective and the detector can filter out the remaining excitation light from fluorescent light.

**Light sources**

Fluorescence microscopy requires intense, near-monochromatic, illumination which some widespread light sources, like halogen lamps cannot provide. Four main types of light source are used, including xenon arc lamps or mercury-vapor lamps with an excitation filter, lasers, supercontinuum sources, and high-power LEDs. Lasers are most widely used for more complex fluorescence microscopy techniques like confocal microscopy and total internal reflection fluorescence microscopy while xenon lamps, and mercury lamps, and LEDs with a dichroic excitation filter are commonly used for widefield epifluorescence microscopes.

**Sample preparation**



A sample of herring sperm stained with SYBR green in a cuvette illuminated by blue light in an epifluorescence microscope. The SYBR green in the sample binds to the herring sperm DNA and, once bound, fluoresces giving off green light when illuminated by blue light.

In order for a sample to be suitable for fluorescence microscopy it must be fluorescent. There are several methods of creating a fluorescent sample; the main techniques are labelling with fluorescent stains or, in the case of biological samples, expression of a fluorescent protein. Alternatively the intrinsic fluorescence of a sample (i.e., autofluorescence) can be used.[1] In the life sciences fluorescence microscopy is a powerful tool which allows the specific and sensitive staining of a specimen in order to detect the distribution of proteins or other molecules of interest. As a result there is a diverse range of techniques for fluorescent staining of biological samples.

**Biological fluorescent stains**

Many fluorescent stains have been designed for a range of biological molecules. Some of these are small molecules which are intrinsically fluorescent and bind a biological molecule of interest. Major examples of these are nucleic acid stains like DAPI and Hoechst (excited by UV wavelength light) and DRAQ5 and DRAQ7 (optimally excited by red light) which all bind the minor groove of DNA, thus labelling the nuclei of cells. Others are drugs or toxins which bind specific cellular structures and have been derivatised with a fluorescent reporter. A major example of this class of fluorescent stain is phalloidin which is used to stain actin fibres in mammalian cells.

There are many fluorescent molecules called fluorophores or fluorochromes such as fluorescein, Alexa Fluors or DyLight 488, which can be chemically linked to a different molecule which binds the target of interest within the sample.

**Advantages of Fluorescence Microscope**

It helps to identify the specific molecules with the help of the fluorescence substances.
Tracing the location of a specific protein in the specimen.
Also for visualizing or capturing the standard pattern how the fluorescent substances affect the cellular structure or tissues at different stages like a **heating stage**.
It offers a magnified and clear image of the cellular molecules in the specimen as compared to the traditional optical microscope.

**Disadvantages**
The greatest disadvantage in fluorescent microscopy is the photobleaching and you cannot focus your specimen for much time at higher magnification (as intense light is required) for more time. And also it needs a quite a sophisticated instrumentation as well as lots of experimental optimization.

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. Photobleaching can severely limit the time over which a sample can be observed by fluorescent microscopy. Several techniques exist to reduce photobleaching such as the use of more robust fluorophores, by minimizing illumination, or by using photoprotective scavenger chemicals.

Fluorescence microscopy with fluorescent reporter proteins has enabled analysis of live cells by fluorescence microscopy, however 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. For example, observing a tissue sample prepared with a fluorescent DNA stain by fluorescent microscopy only reveals the organization of the DNA within the cells and reveals nothing else about the cell morphologies

## 4. Phase contrast microscopy

**Phase contrast microscopy** is an optical microscopy technique that converts phase shifts in light passing through a transparent specimen to brightness changes in the image. Phase shifts themselves are invisible, but become visible when shown as brightness variations.

When light waves travels through a medium other than vacuum, interaction with the medium causes the wave amplitude and phase to change in a manner dependent on properties of the medium. Changes in amplitude (brightness) arise from the scattering and absorption of light, which is often wavelength dependent and may give rise to colors. Photographic equipment and the human eye are only sensitive to amplitude variations. Without special arrangements, phase changes are therefore invisible. Yet, often these changes in phase carry important information.

### History and Background Information

**Frits Zernike**, a Dutch physicist and mathematician, built the first phase contrast microscope in 1938.

It took some time before the scientific community recognized the potential of Zernike's discovery; he won the Nobel Prize in 1953 and the German-based company Zeiss began manufacturing his phase contrast microscope during World War II.

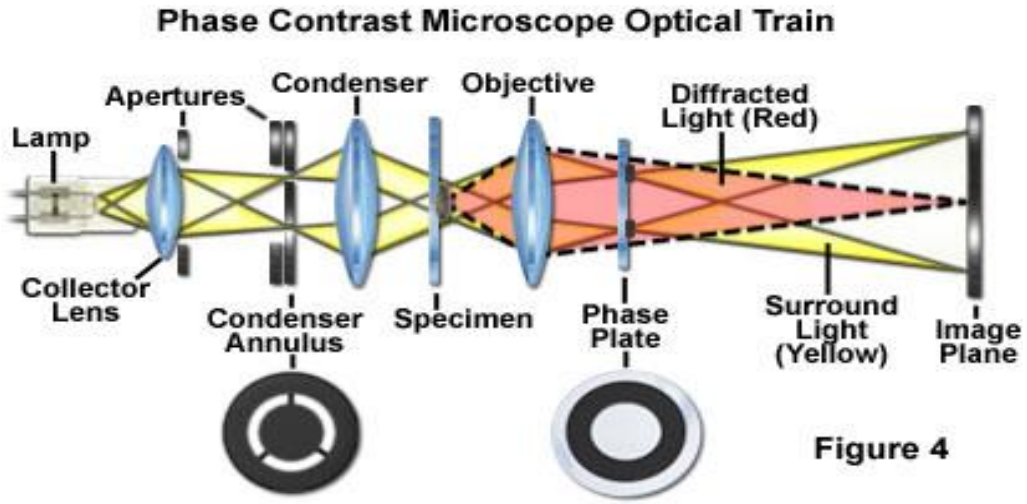
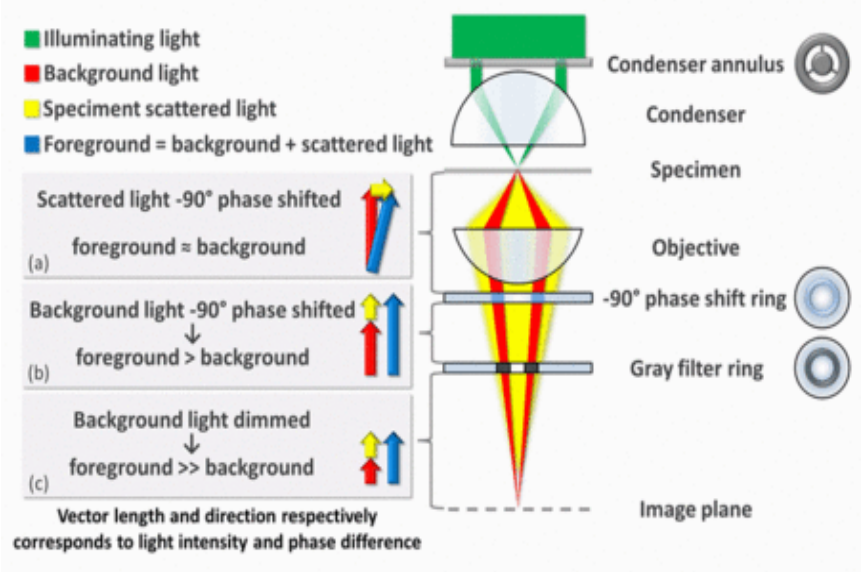



Figure 4

## Working principle




The basic principle to make phase changes visible in phase contrast microscopy is to separate the illuminating background light from the specimen scattered light, which make up the foreground details, and to manipulate these differently.

The ring shaped illuminating light (green) that passes the condenser annulus is focused on the specimen by the condenser. Some of the illuminating light is scattered by the specimen (yellow). The remaining light is unaffected by the specimen and form the background light (red). When observing unstained biological specimen, the scattered light is weak and typically phase shifted by -90° — relative to the background light. This leads to that the foreground (blue vector) and the background (red vector) nearly have the same intensity, resulting in a low image contrast (a).

In a phase contrast microscope, the image contrast is improved in two steps. The background light is phase shifted -90° by passing it through a phase shift ring. This eliminates the phase difference between the background and the scattered light, leading to an increased intensity difference between foreground and background (b). To further increase contrast, the background is dimmed by a gray filter ring (c). Some of the scattered light will be phase shifted and dimmed by the rings. However, the background light is affected to a much greater extent, which creates the phase contrast effect.

The above describes *negative phase contrast*. In its *positive* form, the background light is instead phase shifted by +90°. The background light will thus be 180° out of phase relative to the scattered light. This results in that the scattered light will be subtracted from the background light in (b) to form an image where the foreground is darker than the background.

## Applications in Microscopy

The possible applications of Zernike's phase contrast microscope in microscopy are evident in the fields of molecular and cellular biology, microbiology and medical research.

Specimens that can be observed and studied include live microorganisms such as protozoa, erythrocytes, bacteria, molds and sperm, thin tissue slices, lithographic patterns, fibers, glass fragments and sub-cellular particles such as nuclei and organelles.

**Advantages**

The advantages of the phase contrast microscope include:

- The capacity to observe living cells and, as such, the ability to examine cells in a natural state
- Observing a living organism in its natural state and/or environment can provide far more information than specimens that need to be killed, fixed or stain to view under a microscope
- High-contrast, high-resolution images
- Ideal for studying and interpreting thin specimens
- Ability to combine with other means of observation, such as fluorescence
- Modern phase contrast microscopes, with CCD or CMOS computer devices, can capture photo and/or video images

In addition, advances to the phase contrast microscope, especially those that incorporate technology, enable a scientist to hone in on minute internal structures of a particle and can even detect a mere small number of protein molecules.

**Disadvantages**

Disadvantages and limitations of phase contrast:

- Annuli or rings limit the aperture to some extent, which decreases resolution
- This method of observation is not ideal for thick organisms or particles
- Thick specimens can appear distorted
- Images may appear grey or green, if white or green lights are used, respectively, resulting in poor photomicrography
- Shade-off and halo effect, referred to a phase artifacts
- Shade-off occurs with larger particles, results in a steady reduction of contrast moving from the center of the object toward its edges
- Halo effect, where images are often surrounded by bright areas, which obscure details along the perimeter of the specimen

## Electron microscope

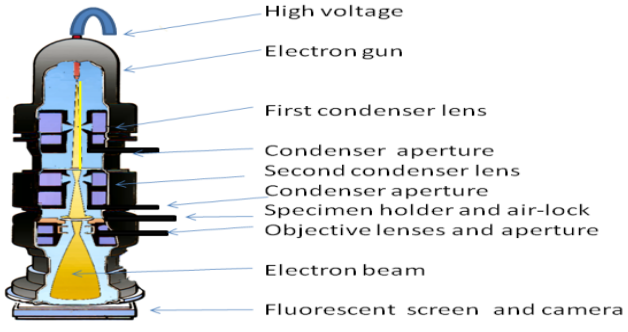
An **electron microscope** is a microscope that uses a beam of accelerated electrons as a source of illumination. As the wavelength of an electron can be up to 100,000 times shorter than that of visible light photons, electron microscopes have a higher resolving power than light microscopes and can reveal the structure of smaller objects. A transmission electron microscope can achieve better than 50 pm resolution[1] and magnifications of up to about 10,000,000x whereas most light microscopes are limited by diffraction to about 200 nm resolution and useful magnifications below 2000x. Transmission electron microscopes use electrostatic and electromagnetic lenses to control the electron beam and focus it to form an image. These electron optical lenses are analogous to the glass lenses of an optical light microscope. Electron microscopes are used to investigate the ultrastructure of a wide range of biological and inorganic specimens including microorganisms, cells, large molecules, biopsy samples, metals, and crystals. Industrially, electron microscopes are often used for quality control and failure analysis. Modern electron microscopes produce electron micrographs using specialized digital cameras and frame grabbers to capture the image.

## History

The first electromagnetic lens was developed in 1926 by Hans Busch.[2] According to Dennis Gabor, the physicist Leó Szilárd tried in 1928 to convince Busch to build an electron microscope, for which he had filed a patent.[3] The physicist **Ernst Ruska** and the electrical engineer **Max Knoll** constructed the prototype electron microscope in 1931, capable of four-hundred-power magnification; the apparatus was the first demonstration of the principles of electron microscopy.[4] Two years later, in 1933, Ruska built an electron microscope that exceeded the resolution attainable with an optical (light) microscope.[4] Moreover, Reinhold Rudenberg, the scientific director of Siemens-Schuckertwerke, obtained the patent for the electron microscope in May 1931.

## Types

### Transmission electron microscope (TEM)




Transmission Electron Microscope

The original form of electron microscope, the transmission electron microscope (TEM) uses a high voltage electron beam to illuminate the specimen and create an image. The electron beam is

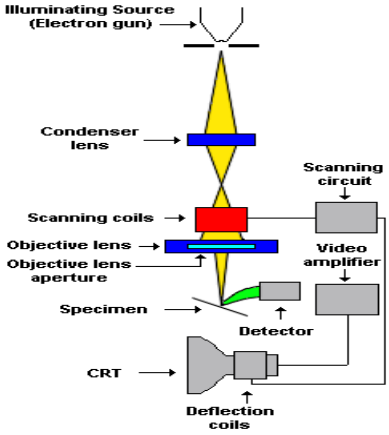
produced by an **electron gun,** commonly fitted with a tungsten filament cathode as the electron source. The electron beam is accelerated by an anode typically at +100 keV (40 to 400 keV) with respect to the cathode, focused by **electrostatic and electromagnetic lenses,** and transmitted through the specimen that is in part transparent to electrons and in part scatters them out of the beam. When it emerges from the specimen, the electron beam carries information about the structure of the specimen that is magnified by the objective lens system of the microscope. The spatial variation in this information (the "image") may be viewed by projecting the magnified electron image onto a fluorescent viewing screen coated with a phosphor or **scintillator material** such as zinc sulfide. Alternatively, the image can be photographically recorded by exposing a photographic film or plate directly to the electron beam, or a high-resolution phosphor may be coupled by means of a lens optical system or a fibre optic light-guide to the sensor of a digital camera. The image detected by the digital camera may be displayed on a monitor or computer.

The resolution of TEMs is limited primarily by spherical aberration, but a new generation of aberration correctors have been able to partially overcome spherical aberration to increase resolution. Hardware correction of spherical aberration for the high-resolution transmission electron microscopy (HRTEM) has allowed the production of images with resolution below 0.5 angstrom (50 picometres)[1] and magnifications above 50 million times.[10] The ability to determine the positions of atoms within materials has made the HRTEM an important tool for nano-technologies research and development.[11]

Transmission electron microscopes are often used in electron diffraction mode. The advantages of electron diffraction over X-ray crystallography are that the specimen need not be a single crystal or even a polycrystalline powder, and also that the Fourier transform reconstruction of the object's magnified structure occurs physically and thus avoids the need for solving the phase problem faced by the X-ray crystallographers after obtaining their X-ray diffraction patterns of a single crystal or polycrystalline powder.

The major disadvantage of the transmission electron microscope is the need for extremely thin sections of the specimens, typically about 100 nanometers. Biological specimens are typically required to be chemically fixed, dehydrated and embedded in a polymer resin to stabilize them sufficiently to allow ultrathin sectioning. Sections of biological specimens, organic polymers and similar materials may require special treatment with heavy atom labels in order to achieve the required image contrast.

**Scanning electron microscope (SEM)**




The SEM produces images by probing the specimen with a focused electron beam that is scanned across a rectangular area of the specimen (raster scanning). When the electron beam interacts with the specimen, it loses energy by a variety of mechanisms. The lost energy is converted into alternative forms such as heat, emission of low-energy secondary electrons and high-energy backscattered electrons, light emission (cathodoluminescence) or X-ray emission, all of which provide signals carrying information about the properties of the specimen surface, such as its topography and composition. The image displayed by an SEM maps the varying intensity of any of these signals into the image in a position corresponding to the position of the beam on the specimen when the signal was generated. In the SEM image of an ant shown below and to the right, the image was constructed from signals produced by a secondary electron detector, the normal or conventional imaging mode in most SEMs.

Generally, the image resolution of an SEM is at least an order of magnitude poorer than that of a TEM. However, because the SEM image relies on surface processes rather than transmission, it is able to image bulk samples up to many centimeters in size and (depending on instrument design and settings) has a great depth of field, and so can produce images that are good representations of the three-dimensional shape of the sample. Another advantage of SEM is its variety called environmental scanning electron microscope (ESEM) can produce images of sufficient quality and resolution with the samples being wet or contained in low vacuum or gas. This greatly facilitates imaging biological samples that are unstable in the high vacuum of conventional electron microscopes.

**Color**

In their most common configurations, electron microscopes produce images with a single brightness value per pixel, with the results usually rendered in grayscale.[12] However, often these images are then colorized through the use of feature-detection software, or simply by hand-editing using a graphics editor. This may be done to clarify structure or for aesthetic effect and generally does not add new information about the specimen.[13]

**Sample preparation**

Materials to be viewed under an electron microscope may require processing to produce a suitable sample. The technique required varies depending on the specimen and the analysis required:

- *Chemical fixation* – for biological specimens aims to stabilize the specimen's mobile macromolecular structure by chemical crosslinking of proteins with aldehydes such as formaldehyde and glutaraldehyde, and lipids with osmium tetroxide.
- *Negative stain* – suspensions containing nanoparticles or fine biological material (such as viruses and bacteria) are briefly mixed with a dilute solution of an electron-opaque solution such as ammonium molybdate, uranyl acetate (or formate), or phosphotungstic acid. This mixture is applied to a suitably coated EM grid, blotted, then allowed to dry. Viewing of this preparation in the TEM should be carried out without delay for best results. The method is important in microbiology for fast but crude morphological identification, but can also be used as the basis for high resolution 3D reconstruction using EM tomography methodology when carbon films are used for support. Negative staining is also used for observation of nanoparticles.
- *Cryofixation* – freezing a specimen so rapidly, in liquid ethane, and maintained at liquid nitrogen or even liquid helium temperatures, so that the water forms vitreous (non-crystalline) ice. This preserves the specimen in a snapshot of its solution state. An entire field called cryo-electron microscopy has branched from this technique. With the development of cryo-electron microscopy of vitreous sections (CEMOVIS), it is now possible to observe samples from virtually any biological specimen close to its native state.[*citation needed*]
- *Dehydration* – or replacement of water with organic solvents such as ethanol or acetone, followed by critical point drying or infiltration with embedding resins. Also freeze drying.
- *Embedding, biological specimens* – after dehydration, tissue for observation in the transmission electron microscope is embedded so it can be sectioned ready for viewing. To do this the tissue is passed through a 'transition solvent' such as propylene oxide (epoxypropane) or acetone and then infiltrated with an epoxy resin such as Araldite, Epon, or Durcupan;[18] tissues may also be embedded directly in water-miscible acrylic resin. After the resin has been polymerized (hardened) the sample is thin sectioned (ultrathin sections) and stained – it is then ready for viewing.
- *Embedding, materials* – after embedding in resin, the specimen is usually ground and polished to a mirror-like finish using ultra-fine abrasives. The polishing process must be performed carefully to minimize scratches and other polishing artifacts that reduce image quality.
- *Metal shadowing* – Metal (e.g. platinum) is evaporated from an overhead electrode and applied to the surface of a biological sample at an angle. The surface topography results in variations in the thickness of the metal that are seen as variations in brightness and contrast in the electron microscope image.
- *Replication* – A surface shadowed with metal (e.g. platinum, or a mixture of carbon and platinum) at an angle is coated with pure carbon evaporated from carbon electrodes at right angles to the surface. This is followed by removal of the specimen material (e.g. in an acid bath, using enzymes or by mechanical separation[19]) to produce a surface replica

that records the surface ultrastructure and can be examined using transmission electron microscopy.

- *Sectioning* – produces thin slices of specimen, semitransparent to electrons. These can be cut on an ultramicrotome with a diamond knife to produce ultra-thin sections about 60–90 nm thick. Disposable glass knives are also used because they can be made in the lab and are much cheaper.
- *Staining* – uses heavy metals such as lead, uranium or tungsten to scatter imaging electrons and thus give contrast between different structures, since many (especially biological) materials are nearly "transparent" to electrons (weak phase objects). In biology, specimens can be stained "en bloc" before embedding and also later after sectioning. Typically thin sections are stained for several minutes with an aqueous or alcoholic solution of uranyl acetate followed by aqueous lead citrate.[20]
- *Freeze-fracture or freeze-etch* – a preparation method particularly useful for examining lipid membranes and their incorporated proteins in "face on" view. The fresh tissue or cell suspension is frozen rapidly (cryofixation), then fractured by breaking or by using a microtome while maintained at liquid nitrogen temperature. The cold fractured surface (sometimes "etched" by increasing the temperature to about −100 °C for several minutes to let some ice sublime) is then shadowed with evaporated platinum or gold at an average angle of 45° in a high vacuum evaporator. A second coat of carbon, evaporated perpendicular to the average surface plane is often performed to improve stability of the replica coating. The specimen is returned to room temperature and pressure, then the extremely fragile "pre-shadowed" metal replica of the fracture surface is released from the underlying biological material by careful chemical digestion with acids, hypochlorite solution or SDS detergent. The still-floating replica is thoroughly washed free from residual chemicals, carefully fished up on fine grids, dried then viewed in the TEM.
- *Ion beam milling* – thins samples until they are transparent to electrons by firing ions (typically argon) at the surface from an angle and sputtering material from the surface. A subclass of this is focused ion beam milling, where gallium ions are used to produce an electron transparent membrane in a specific region of the sample, for example through a device within a microprocessor. Ion beam milling may also be used for cross-section polishing prior to SEM analysis of materials that are difficult to prepare using mechanical polishing.
- *Conductive coating* – an ultrathin coating of electrically conducting material, deposited either by high vacuum evaporation or by low vacuum sputter coating of the sample. This is done to prevent the accumulation of static electric fields at the specimen due to the electron irradiation required during imaging. The coating materials include gold, gold/palladium, platinum, tungsten, graphite, etc.
- *Earthing* – to avoid electrical charge accumulation on a conductive coated sample, it is usually electrically connected to the metal sample holder. Often an electrically conductive adhesive is used for this purpose.

## Disadvantages

Electron microscopes are expensive to build and maintain, but the capital and running costs of confocal light microscope systems now overlaps with those of basic electron microscopes. Microscopes designed to achieve high resolutions must be housed in stable buildings (sometimes underground) with special services such as magnetic field cancelling systems.

The samples largely have to be viewed in vacuum, as the molecules that make up air would scatter the electrons. An exception is the environmental scanning electron microscope, which allows hydrated samples to be viewed in a low-pressure (up to 20 Torr or 2.7 kPa) and/or wet environment.

Scanning electron microscopes operating in conventional high-vacuum mode usually image conductive specimens; therefore non-conductive materials require conductive coating (gold/palladium alloy, carbon, osmium, etc.). The low-voltage mode of modern microscopes makes possible the observation of non-conductive specimens without coating. Non-conductive materials can be imaged also by a variable pressure (or environmental) scanning electron microscope. Small, stable specimens such as carbon nanotubes, diatom frustules and small mineral crystals (asbestos fibres, for example) require no special treatment before being examined in the electron microscope. Samples of hydrated materials, including almost all biological specimens have to be prepared in various ways to stabilize them, reduce their thickness (ultrathin sectioning) and increase their electron optical contrast (staining).

## Applications

Semiconductor and data storage

- Circuit edit[24]
- Defect analysis[25]
- Failure analysis[26]

**Biology and life sciences**

- Cryobiology[27]
- Cryo-electron microscopy[28]
- Diagnostic electron microscopy[29]
- Drug research (e.g. antibiotics)[30]
- Electron tomography[31]
- Particle analysis[32]
- Particle detection[33]
- Protein localization[34]
- Structural biology[28]
- Tissue imaging[35]
- Toxicology[36]
- Virology (e.g. viral load monitoring)[37]

Materials research

- Device testing and characterization[38]
- Dynamic materials experiments[39]
- Electron beam-induced deposition[40]
- Materials qualification[41]
- Medical research[30]
- Nanometrology[42]
- Nanoprototyping[43]

Industry

- Chemical/Petrochemical[44]
- Direct beam-writing fabrication[45]
- Food science[46]
- Forensics[47]
- Fractography[48]
- Micro-characterization[49]
- Mining (mineral liberation analysis)[50]
- Pharmaceutical QC[51]