Microbiological & Immunological Communications
DOI: doi.org/10.55627/mic.003.01.0567

Review Article

Bacterial Morphology and Microscopic Advancements: Navigating from Basics to Breakthroughs

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Abstract

Bacterial morphology is critical in determining how these organisms function and behave. This study delved into the intriguing world of bacterial morphologies, offering thorough research regarding microorganisms' variable shapes and structures. It sheds light on the distinct traits and functions associated with their morphologies. The study highlights the most common bacterial morphologies, including spirals, helices, and flat-wave structures. Each morphology revealed the structural traits that separate one from another. Moreover, a particular focus was placed on emerging advancements in the field, from conventional to recent advances in microscopic technologies that provided unique insights into microbial architecture. Furthermore, the review emphasized the importance of bacterial surface appendages, including flagella and pili, influencing microbial behavior and interactions. For instance, the corkscrew-like motion of helical bacteria is investigated in motility, emphasizing its importance in the survival and adaptability of specific species. Furthermore, in the realm of microscopic technologies, various options exist. However, recent literature revealed that scanning electron microscopy (SEM), field-emission scanning electron microscopy (FESEM), and transmission electron microscopy (TEM) stand out as the most advanced microscopic techniques. Specifically, advancements such as super-resolution microscopy techniques, including structured illumination microscopy (SIM) and stochastic optical reconstruction microscopy (STORM), have revolutionized our understanding of pathogenesis and drug delivery. In conclusion, this review strengthens our understanding of bacterial morphologies while emphasizing the importance of a diverse approach to investigating microbes. The combination of traditional knowledge and cutting-edge microscopy opens up new possibilities for discovery, promising a better understanding of bacterial shape and its consequences in scientific and medical fields.

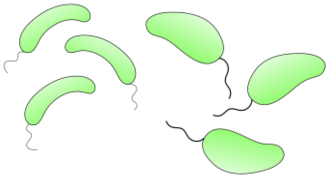
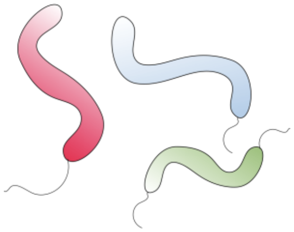
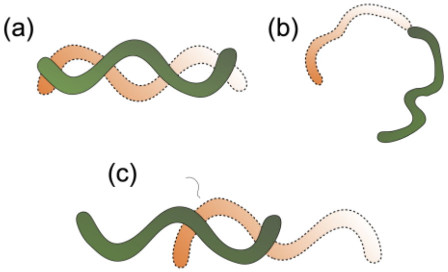
Keywords: Bacterial morphology, microscopy, microscopic techniques

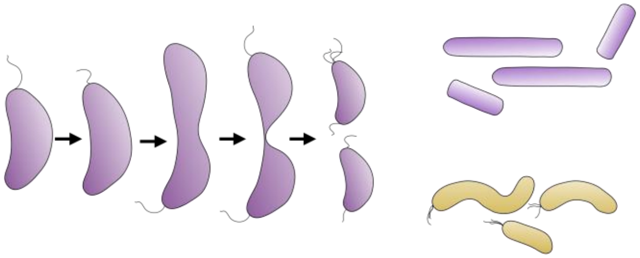
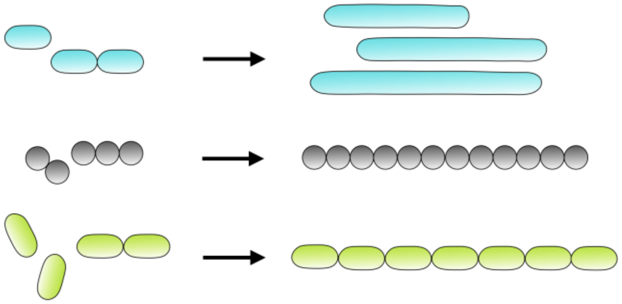
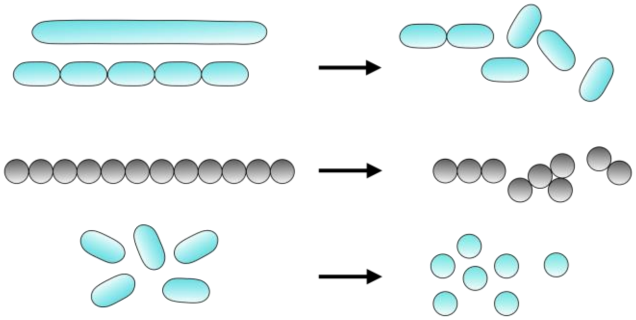
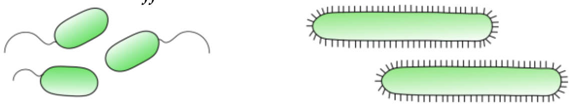
1. Introduction

In general, bacteria are tiny living things with sizes ranging from 0.5 to 5.0 μm in length, with the smallest members, such as mycoplasma, measuring only 0.3 μm(Guijuan, 2020). Furthermore, the entire study of bacterial structure and function is called bacteriology. The use of microscopy represents an essential turning point in this endeavor(*Atlas of Oral Microbiology*, 2015; Möckl, 2020). This technique is regarded as a significant tool for diagnosing and measuring the surfaces of bacterial cells. The invention of the microscope constituted a watershed moment in science, allowing scientists to view organisms and objects that were too tiny to be seen with the naked eye. In general, microscopy is the technical field that studies microscopic materials such as microorganisms. Bacteriology and microscopy are inextricably linked, with microscopy essential in studying bacterial morphological traits. Microscopy in bacteriology uses a variety of methods, including bright field microscopy (BFM), dark field microscopy (DFM), Atomic Force Microscopy (AFM), Scanning Electron Microscopy (SEM), and Transmission Electron Microscopy (TEM). Each of these technologies provides unique viewpoints and insights into the intricate world of bacteria, considerably improving our understanding of their morphology, structure, and behavior(J. Sheikh, et al, 2021; Tardelli JDC, 2023; Xin, 2023; Zhao Z., 023). Furthermore, microscopy has evolved as a valuable tool for identifying and characterizing causative pathogens and analyzing samples for disease diagnosis in organisms. This potent instrument further allows for the microscopic detection of organisms, especially when stained with different antibodies labeled with fluorescent dyes or other markers. This novel approach has proven extremely useful for the particular identification of bacteria. With such technologies, researchers can improve the precision and accuracy of bacterial identification by using antibodies with specialized affinities for bacterial components and fluorescent dyes or markers. The advancement of new labeling techniques has significantly enhanced our capacity to microscopically detect stained organisms, thereby improving our ability to promptly and effectively localize and diagnose bacterial infections(Ziyue, 2023). This increased precision is critical when determining the number of bacterial cells and assessing their biomass. Using electron microscopy, research can get remarkable insights into the delicate intricacies of bacterial shape and physiology, contributing to a more thorough understanding of these microorganisms(Hamouda, 2023; Sara, 020). Furthermore, continual advances in microscopy techniques have substantially impacted various sectors, including diagnosis, medication discovery, and disease target identification. These breakthroughs have enabled researchers to visualize and analyze bacterial structures in unprecedented depth, allowing for more accurate infection diagnosis and the discovery of new medications and disease targets. In this review, we aim to investigate the crucial role of microscopy in expanding our understanding of bacterial morphology and its numerous applications in these critical fields of research.

Therefore, this study thoroughly analyzed and comprehensively elucidated the various morphologies exhibited by bacteria, with the main emphasis on determining the distinct traits and functions associated with each form. By delving into the complexities of bacterial structures, we aimed to provide a more nuanced understanding of the relationship between morphology and microbial behavior. The study will additionally explore existing imaging methods, focusing on their effectiveness in capturing and analyzing bacterial morphology. Through this research, we aim to contribute to advancing knowledge in microbiology, potentially uncovering novel insights that could have implications for various scientific and medical applications.

Table 1. Unveils the diverse bacterial morphologies and their unique features and functions

Morphological Features/ Shapes	Common Microorganisms	Associated Features and Function	Reference
Curvature 	• *Caulobacter crescentus* (*C. crescentus*) • *Vibro cholerae* (*V. cholerae*) • *Vibro parahaemolyticus* (*V. parahaemolyticus)*	This adaptation allows bacteria to flourish and form robust colonies in situations with a moderate flow of water. Cellular curvature improves their ability to navigate through viscous fluids.	(Robertson, 2005)
Helical 	• *Helicobacter pylori (H. pylori)* • *Campylobacter jejuni* (*C. jejuni*) • *Spirochetes sp.*	Certain bacteria have a specific advantage due to their helical form, which increases torque. This increased torque has the potential to increase the pace of these bacteria and aid in their escape from viscous liquids. The bacterium's helical structure facilitates the corkscrew-like motion required for movement.	(Choi, 2022)
Flat-Wave  	• *Borrelia burgdorferi* (*B. burgdorferi*) • *Treponema pallidum* (*T. pallidum*)	• Wriggling • Lunging and • Translocating	(Nakamura S., 2022)
Heterogeneity in Shape	• *C. cresentus* • *H. pylori* • *Myobacterium tuberculosis* (*M. tuberculosis*)	Bacterial asymmetric growth and division promote directional motility, develop daughter cells with varied cell fates, and result in heterogeneous populations with varying susceptibility to antibiotics and other stressors.	(Henry H. M., 2022)

			
Filamentation 	- *Legionella pneumophila* (*L. pneumophila*) - *Streptococcus pneumoniae* (*S. pneumoniae*) - *Escherichia coli* (*E. coli*)	Bacteria can use a variety of techniques to avoid phagocytosis-mediated death. They may facilitate slow, ligand-mediated uptake and invasion, as well as improve adhesion to host surfaces or cells. These methods let the bacteria resist immune responses and form infections in the host.	(2022)
Size Minimization 	- *Moraxella catarrhalis* (*M. catarrhalis*) - *Neisseria meningitidis* (*N. meningitidis*) - *Salmonella typhimurium* (*S. typhimurium*) - *S. pneumoniae*	Bacterial lineages found on mucosal surfaces show a trend of size reduction, shifting from rod-shaped to cocci-shaped forms.	(Dalia AB., 2011)
Swarm Cell Differentiation 	- *Pseudomonas aeruginosa* (*P. aeruginosa*) - *Proteus mirabilis* (*P. mirabilis*)	Surface sensing through rotational inhibition of the polar flagellum triggers genetic reprogramming events that can lead to cell elongation and the production of proteins that promote swarming motility.	(Kearns DB., 2010)

2.1. Background and Significance

In the history of microbiology, William J. Costerton formally invented the term "biofilm" in 1978. Costerton, a pioneering figure in the field, gave this name to surface-attached microbial agglomerations(Gurrerro, 2023). These biofilms are groups of microbes attached to surfaces and embed themselves in a self-produced matrix of extracellular polymeric substances (EPS). Costerton's invention of the phrase 'biofilm' was a watershed moment, providing a concise and complete title for a phenomenon that has long captivated scientists researching microbial interactions with surfaces. This phrase has now become an essential part of understanding and discussing the complex and resilient microbial communities that grow on varied surfaces in a variety of settings(Yadav P, 2020). In subsequent periods, he refined the definition of biofilm to include additional features such as the host's function and the three-dimensional (3-D) architecture of these microbial communities. This evolved to refer to a complex assemblage of microorganisms that are adhered to a surface or interface. Essentially, these microbial populations are surrounded by an EPS produced from microbial and host sources. Furthermore, the microorganisms within this matrix are organized in a 3-D design. This modified description addressed the intricate character of biofilms, considering not only surface attachment and EPS composition but also the host's engagement and the microbial community's spatial organization. Since then, this comprehensive description has been critical in expanding our understanding of biofilm formation and its significance in various biomedical and ecological contexts(Costerton J.W., 1995). The bacterial species that inhabit biofilms exhibit an extraordinary degree of cooperation, acting as complex multicellular organisms(Penesyan, 20211; Ruchika, 2021). The EPS in biofilms is a complex and multifaceted material that includes proteins, lipids, nucleic acids, polysaccharides, and even metals(Syed, 2023). 'Matrixome' is the term used to describe this complex collection of chemically and functionally varied biomolecules found inside the extracellular polymer(Flemming, 2023). The unique traits of biofilm behavior are largely attributed to the matrixome. The National Institutes of Health (NIH) reports that bacterial biofilms are potentially the cause of up to 75% of human infectious disorders, especially those involving implants or tissues(Yadav M.K., 2020). Furthermore, biofilms are responsible for roughly 8.9 million cases of healthcare-associated infections in the European Union and the European Economic Area each year(Suetens et al., 2018). Frequently, these infections are recurring and resistant to antibiotics(Vestby L.K., 2020). This resistance is due to the unique properties of EPS, which protect resident bacteria against the effects of both host immunity and antimicrobial drugs(Li Y., 2020). In the current setting, creating or identifying molecules with anti-biofilm capabilities that can effectively reduce and remove biofilm-associated infections is of the utmost significance. In the pursuit of such investigations, the use of diverse microscopy techniques is required to acquire a better understanding of the intricate elements of biofilms. These approaches are necessary for an in-depth knowledge of biofilm ultrastructure, three-dimensional organization, cell population behaviour, and responses to pharmacological treatments. Researchers can use a variety of microscopy approaches to uncover the nuanced features required for developing efficient strategies

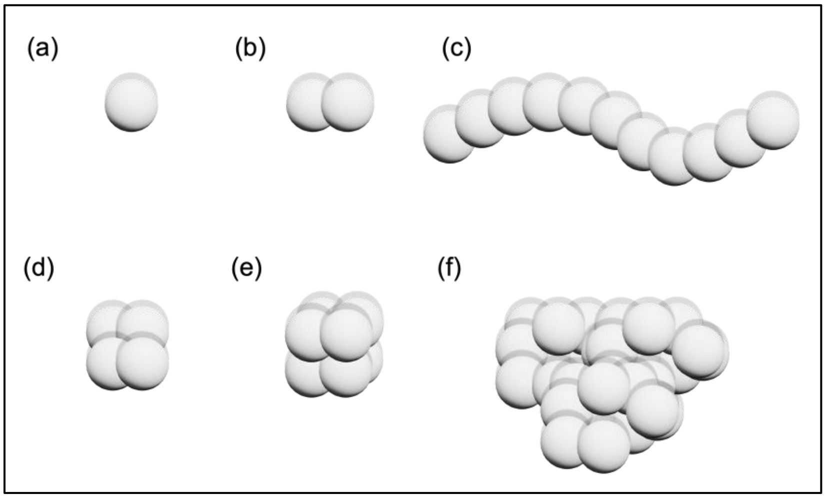


Figure 1. Graphical illustration of the type of cocci-shaped morphologies: (a) Monococcus, (b) Diplococcus, (c) Streptococcus (d) Tetacoccus, (e) Sarcina and (f) Staphylococcus.

to treat and remove biofilm-related diseases(Papa R., 2020).

2. Bacterial Morphology: Basics and Significance

2.1 Exploring the Diverse Functions of Bacterial Structures

Bacteria have various cell body shapes, including spherical forms (cocci) and elongated configurations (bacilli) with different curvatures and spiral patterns. Some bacteria can take on strange shapes, such as star-shaped. Moreover, bacteria can produce numerous appendages, such as pili or flagella, that differ in form, length, width, and positioning relative to the cell body. Notably, bacteria can undergo morphological changes during their life cycle or in reaction to external stimuli. Much of our understanding of the mechanisms underlying various bacterial morphologies has come from studying model organisms in controlled laboratory environments. However, as researchers investigate harmful bacteria in infection models, they are increasingly observing morphological variances. Mutants with altered colonization or pathogenicity properties are frequently associated with changes in bacterial morphology(Yang DC, 2016). Table 1 depicts a detailed exploration of several typical bacterial morphologies, highlighting their distinguishing traits and functions. The thorough analysis of each morphology provided a complete understanding of the microbial world, emphasizing the importance of form in regulating bacterial behavior and adaptivity.

2.2 A Brief Overview of Shapes and Structures in Microbial Worlds

2.2.1 Round or Cocci Shape Bacterial Morphology

The most straightforward bacterial cell shape is that of a sphere. Bacteria having this form are classified as either cocci (totally circular, as in staphylococci and micrococci) or ovo-coccoid (a bit shaped like an egg, as in streptococci or lacto-cocci). Several bacterial pathogens fall into these morphological groups, including the Gram-positive bacteria *S. aureus* (coccoid) and *S.*

Figure 2. Graphical illustration of the rod-shaped morphology

pneumoniae (ovo-coccoid), as well as the Gram-negative coccoid pathogens Neisseria gonorrhoeae and N. meningitidis. Substantial study on *S. aureus* (Figure. 1) and *S. pneumoniae* has contributed significantly to our understanding of bacterial processes in cocci because of the comprehensive investigations undertaken by diverse research groups over time(Li, 2018; J. Sheikh et al., 2021). Coccoid bacteria, such as *S. aureus*, use a cell wall growth strategy that relies only on cell division machinery. This process is essential for creating septal peptidoglycan (PG), a critical component of bacterial cell walls. Septal PGs form at the division site during cell division. On the other hand, ovo-coccoid bacteria, such as *S. pneumoniae*, use a more complex method to create new cell walls(Sashi, 2023). In addition to the cell division machinery, ovo-coccoid bacteria use an elongation complex to produce peripheral PG.

Among the numerous morphologies of cocci shapes (as depicted in Figure 1), diplococcus is a prevalent form of bacteria. This parasitic bacterium makes the respiratory systems of humans and several other mammals its home. These bacteria grow to be between 0.5 and 1.25 µm in size(Tembhurne, 2023), nd they are more likely to multiply in liquid settings when found alone or in pairs. This bacterium's cells take on an almost spherical shape, which adds to its unique morphology. This adaptation makes it possible for the organism to live and move about in the complex respiratory system milieu, where it may cause infections or other health problems(Marco, 2024). Among the discrete cocci shaped, *Micrococcus flavus* (*M. flavus*) is among the prevalent monococcus bacteria. Tetracoccus is a species of bacteria that divides into two planes at right angles to one another and is identified by its arrangement of four round cells. *Gaffkya tetragena* is an excellent example of tetracoccus as it stands out due to its unique four-cell arrangement and perpendicular orientation, which highlights the range of structural variants seen in the world of cocci-shaped bacteria. In the case of *Sarcina*, cellular division occurs in three planes, resulting in a unique cube-like configuration comprising either eight or sixteen cells. Despite the multiple divisions, the cells maintain a regular and organized shape. A common example of such a type is *Sarcina lutea* (*S. lutea*)(Lam-Himlin D, 2011). This distinct cubical arrangement of cells in Sarcina showcases a specific pattern of growth, contributing to the characteristic morphology associated with this bacterial genus. Cell division in *Sarcina* forms a distinct cube-like structure in three planes. The cells maintain their regular, structured morphology even after being divided several times. *S. lutea* serves as an illustration of this phenomenon. A common example of such

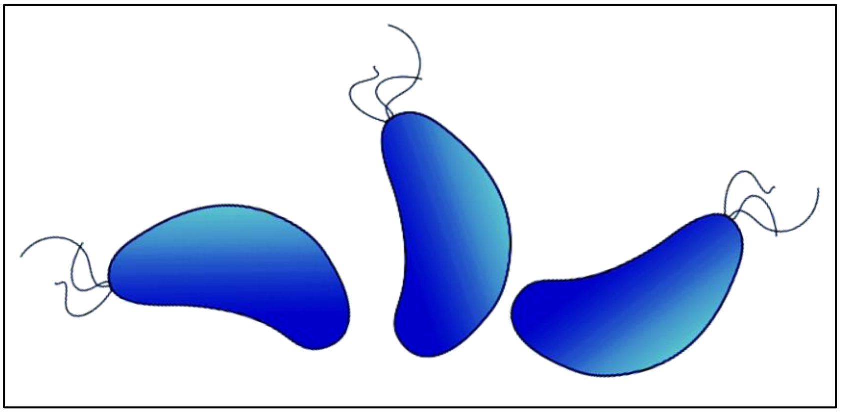


Figure 3. Graphical illustration of the crescent-shaped morphology

type is *S. lutea*. This unique, cubic cell structure displays a particular growth pattern and adds to the genus's distinctive morphology. In Streptococcus, on the other hand, cells divide repeatedly within a single plane, forming a chain of linked cells. Because of its unique mechanism of division, which produces a linear arrangement(Skye M. B.).

2.2.2 Rod Shape Bacterial Morphology

Two exceptionally well-researched rod-shaped bacteria are the Gram-positive *Bacillus subtilis* (*B. subtilis*) and the Gram-negative *E. coli*, as illustrated in Figure 2. Studies on these model organisms have substantially contributed to our knowledge of the formation of cell shapes in rod-shaped and non-bacillary bacteria. Most rod-shaped bacteria have their morphology due to peripheral sidewall elongation and septal cell wall development.

On the other hand, some rod-shaped bacteria, such as mycobacteria, agrobacteria, and coryne bacteria, have a unique way of growing that involves elongation only at the poles of the cells. The diversity of growth methods highlights the complexity and versatility of bacterial cell morphologies(Daniel RA, 2003; Thanky NR, 2007). *P. mirabilis*, a Gram-negative rod-shaped bacterium associated with urinary tract infections, shows a significant link between bacillary morphology and multicellular swarming movement. According to research, *P.* mirabilis' distinctive rod form aids in the coordinated movement of numerous cells, a phenomenon known as swarming motility(Jessica N. S., 2016).

2.2.3 Crescent Shape Bacterial Morphology

The organism *C. crescentus*, typically found in freshwater habitats such as streams and lakes, is one of the most widely recognized instances of a bacteria obtaining its distinctive cell shape. Several independent natural isolates of *C. crescentus* have a similar 'crescent' form, implying that the curved cell structure (Figure 3) provides a specific evolutionary advantage in their natural habitat(Yue Liu., 2024). The bacterium, as the name implies, has a peculiar crescent or vibrionic morphology regulated by a single protein called crescentin. In the case of *C. crescentus*, the presence and activity of crescentin are critical in structuring the bacterium's cell structure (Figure 4), contributing to its distinct and easily identifiable appearance. This unique example demonstrates the importance of

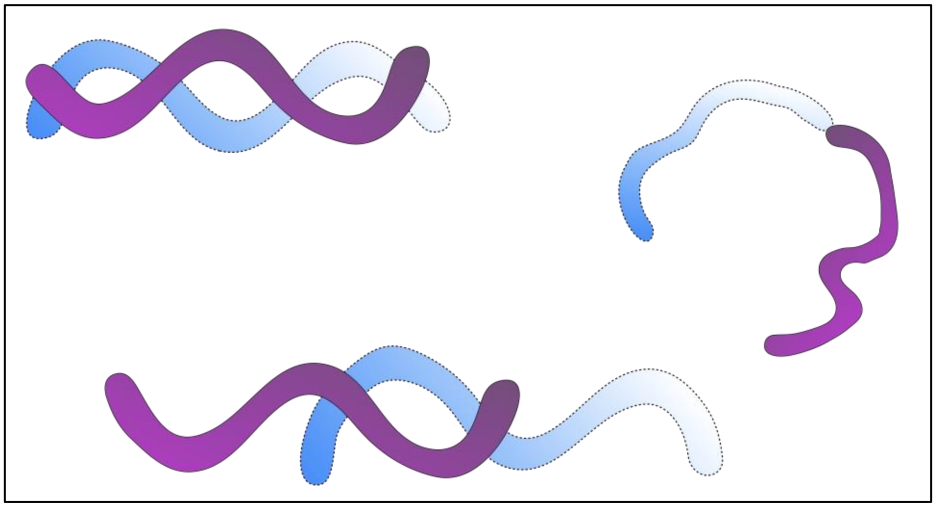


Figure 4. Graphical illustration of the helical and flat-wave morphology

individual proteins in determining the many morphologies observed in bacterial species, offering light on the complex mechanisms behind bacterial cell shape formation(Daniel C., 2018; Surovtsev, 2018). Persat and colleagues (Persat A, 2014) developed the first experimentally confirmed model to explain why *C. crescentus* has its distinctive curved-cell shape. Their research focused on the role of crescentin, a protein that influences the bacterium's morphology, and most likely examined how it interacts with the cell's structural components. This model considerably improved the understanding of the molecular and physiological events that contribute to *C. crescentus* unique form, shedding light on larger principles of bacterial cell morphology.

2.2.4 Helical and Flat-wave Bacterial Morphology

Various bacteria have independently developed helical forms throughout their evolutionary history, resulting in unique groupings of bacterial pathogens. *Spirochetes* such as *Treponema, Spirochaeta, Leptospira*, and *Borrelia* spp. are examples, as are proteobacteria like Helicobacter spp. (both gastric and nongastric) and the foodborne pathogen *C. jejuni*. These microbes are accountable for various infections in people and animals. Studies on both helical proteobacteria and spirochetes have demonstrated that a loss of motility and the distinctive helical cell shape significantly decreases the organisms' colonization capability(Kai., 2022; Nedeljković M., 2021). It is worth noting that helical and planar-wave cell morphologies are prevalent in Gram-negative bacteria. However, a significant exception to this pattern is the helical structure of Streptomyces aerial hyphae, which is caused by polar growth(Matthew P. Z., 2022).

Spirochetes have evolved alternate methods of forming helical and flat-wave morphologies. Periplasmic flagella (PF) relates motility with cell shape in some spirochetes. *B. burgdorferi*, the bacterium that causes Lyme disease and is spread by ticks such as *Ixodes scapularis* (*I. scapularis*), has flat-wave or planar-wave morphology due to the inherent wave-form structure of its periplasmic flagella. In *B. burgdorferi*, removing the periplasmic flagella results in the loss of motility as well as the distinctive

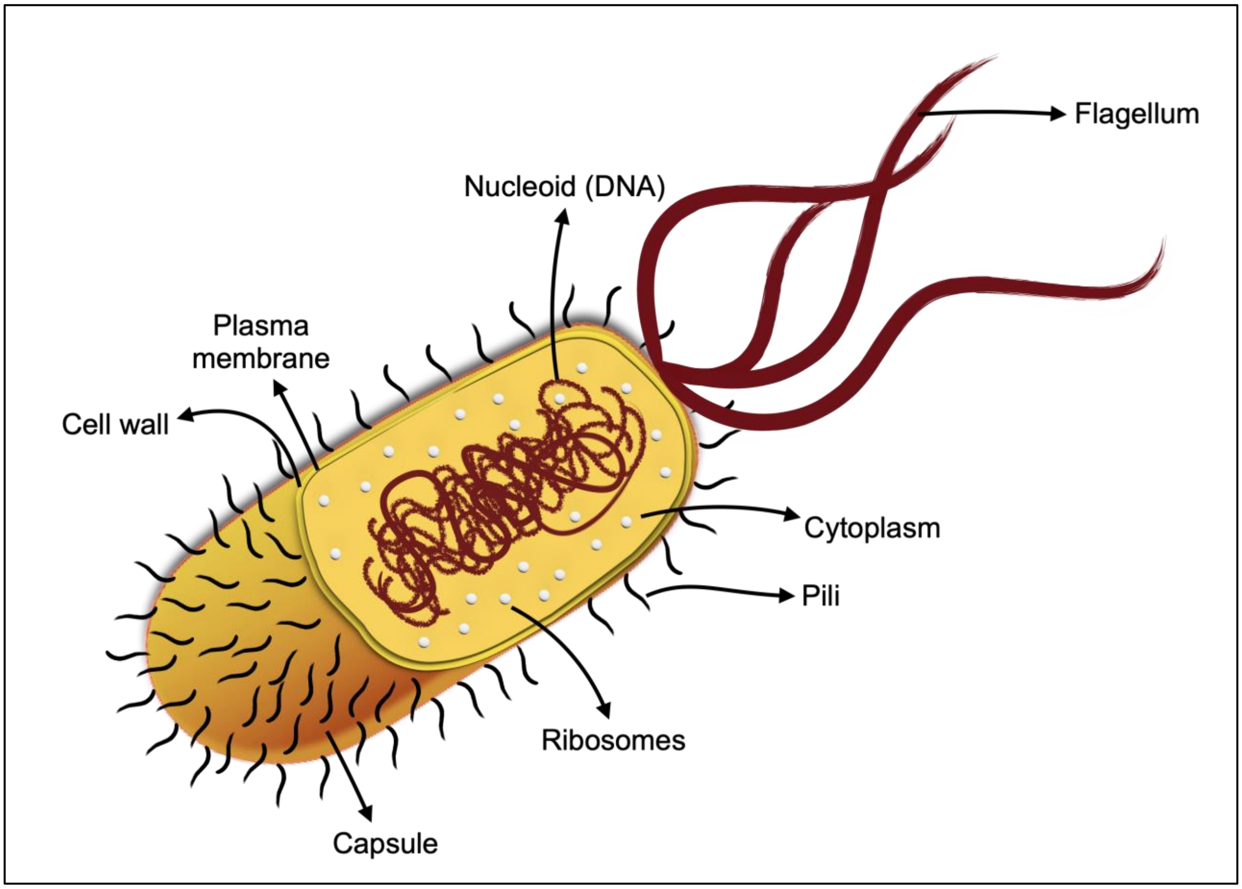


Figure 5. Graphical illustration of the bacterial flagellum and pili.

helical form(Kai, 2020). As the flagella rotate in the periplasmic region, they cause deformation in the cell envelope and peptidoglycan (PG) layer, resulting in a propagating wave along the cell axis. This wave displaces fluid and propels the bacteria forward. Biophysical modeling studies of *B. burgdorferi* PF reveal two unique waveforms, one of which contributes to the bacterium's flat or planar-wave shape(Dombrowski C, 2009). The postulated functional significance of the helical cell shape relies on the concept that it improves movements in viscous fluids or gels, possibly through a corkscrew or burrowing model of motility(Bansil, 2023).

2.3 Exploring Bacterial Surface Appendages and Their Multifaceted Roles

2.3.1 Flagella and Pili

Certain bacterial species have two types of surface appendages (as depicted in Figure 5): flagella, which act as motility organs, and pili (also known as fimbriae, from Latin "fringes" or "hair"). Flagella are found in Gram-positive and Gram-negative bacteria, and their presence or absence can be helpful for identification. For example, they are prevalent in many bacilli species but rare in cocci.

Pili, on the other hand, are most commonly linked with Gram-negative bacteria, with only a few Gram-positive organisms, such as *E. coli(Vinay, 2021)*, possessing them. Interestingly, some bacteria have both flagella and pili, demonstrating a combination of both surface appendages that

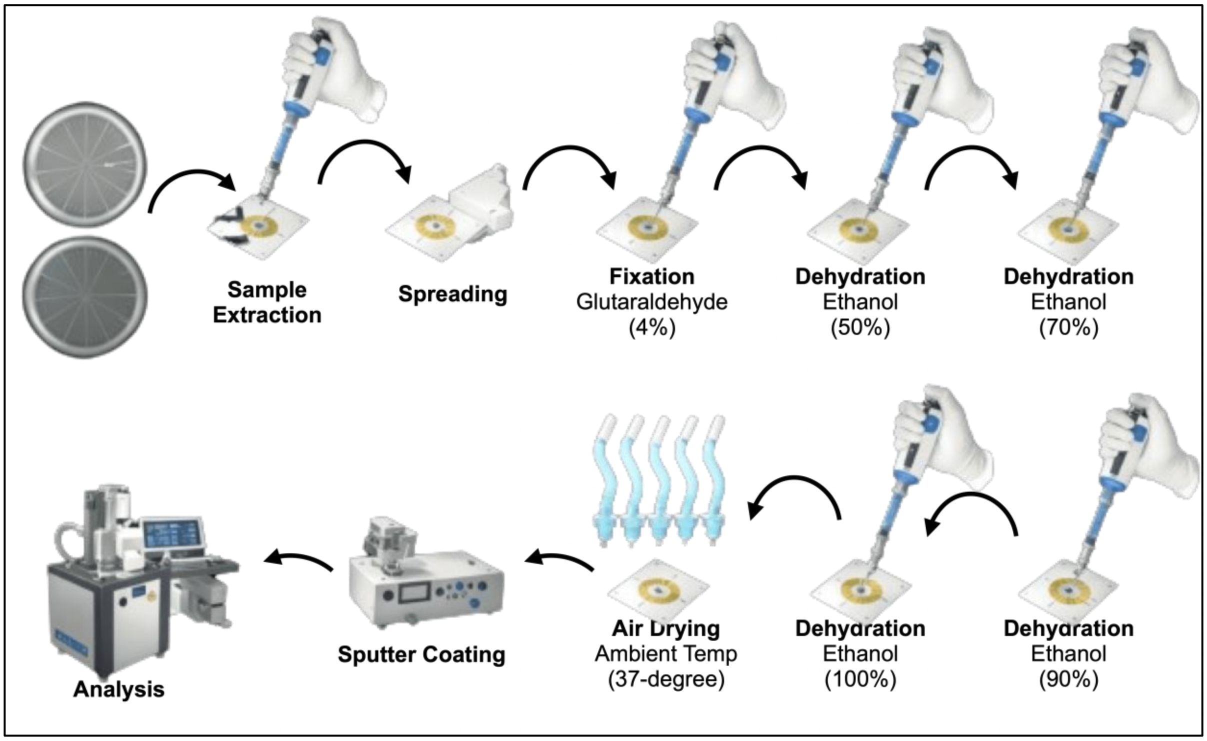


Figure 6. Preparation of sample prior to analysis under imaging techniques

aid in motility, adhesion, and general functionality.

Bacterial flagella consist of long filamentous appendages ranging from 3 to 12 μm in length and 12 to 15 nm in diameter(Eric, 2021). These appendages are created by assembling protein subunits into cylindrical structures with hollow centers. The flagellum is made up of three essential components: (a) the extended filament, which is located externally on the cell surface; (b) the hook structure at the filament's end; and (c) the basal body, which is anchored by the hook and is responsible for the flagellum's motility. The basal body, which consists of a rod and one or two pairs of discs, travels through the outer wall and membrane structures. The bacterial cell moves forward by rotating the basal body anticlockwise, which causes a swirling motion in the helically coiled filament(Aizawa, 2024).

The terms pili and fimbriae are occasionally employed interchangeably to describe the slender, hair-like projections on the surface of many Gram-negative bacteria(Rachel A, 2021), and the protein components of pili are known as pilins. Pili have a more solid look than flagella. In some organisms, such as Shigella species and *E. coli*, pili are abundantly scattered across the cell surface, with up to 200 per cell. Pili in *E. coli* strains are remarkably variable, with two primary types: numerous short common pili and a small number (varying from one to six) of extremely lengthy pili known as sex pili. Sex pili are distinguished by their ability to bind to male-specific bacteriophages. Many enteric bacteria have pili, which give them adhesive qualities and allow them to cling to different epithelial surfaces, red blood cells (producing hemagglutination), and yeast and fungal cell surfaces. Piliated cells' adhesive qualities play a crucial role in

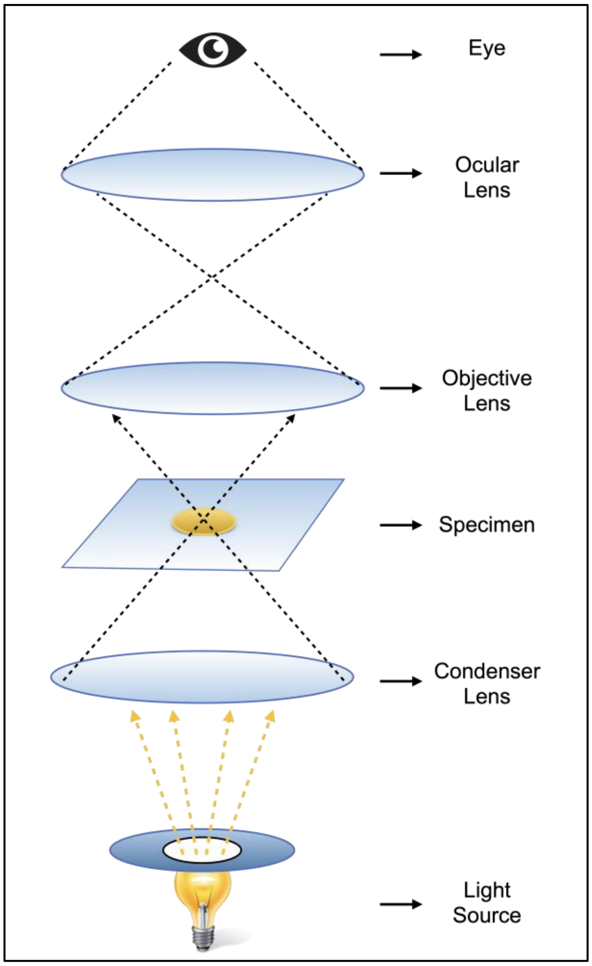


Figure 7. The working mechanism of a light microscope

bacterial colonization of epithelial surfaces and are thus referred to as colonization factors(Drame, 2021).

2.3.2 Capsule Shape

Bacteria generate capsules (Figure 5), which surround the cell with a dense coating of viscous gel up to 10 µm thick(Rhodesherdelin, 2020). While some species lack a well-defined capsule, they may have loose, shapeless slime layers outside the cell wall or cell envelope. *Streptococcus mutans* (*S. mutans*), the principal bacteria in dental plaque, can synthesize glucans from sucrose. It's crucial to remember that not every bacterial species produces capsules. However, the capsules of encapsulated diseases frequently play an essential role in determining pathogenicity. Encapsulated species exist in both Gram-positive and Gram-negative bacteria. In both groups, the bulk of capsules comprises high-molecular-weight viscous polysaccharides, which produce a thick gel that remains outside the cell wall or envelope(Ming, 2020).

2.3.3 Cell Wall

Most Gram-positive bacteria have a relatively thick, continuous cell wall known as the sacculus, which ranges in thickness

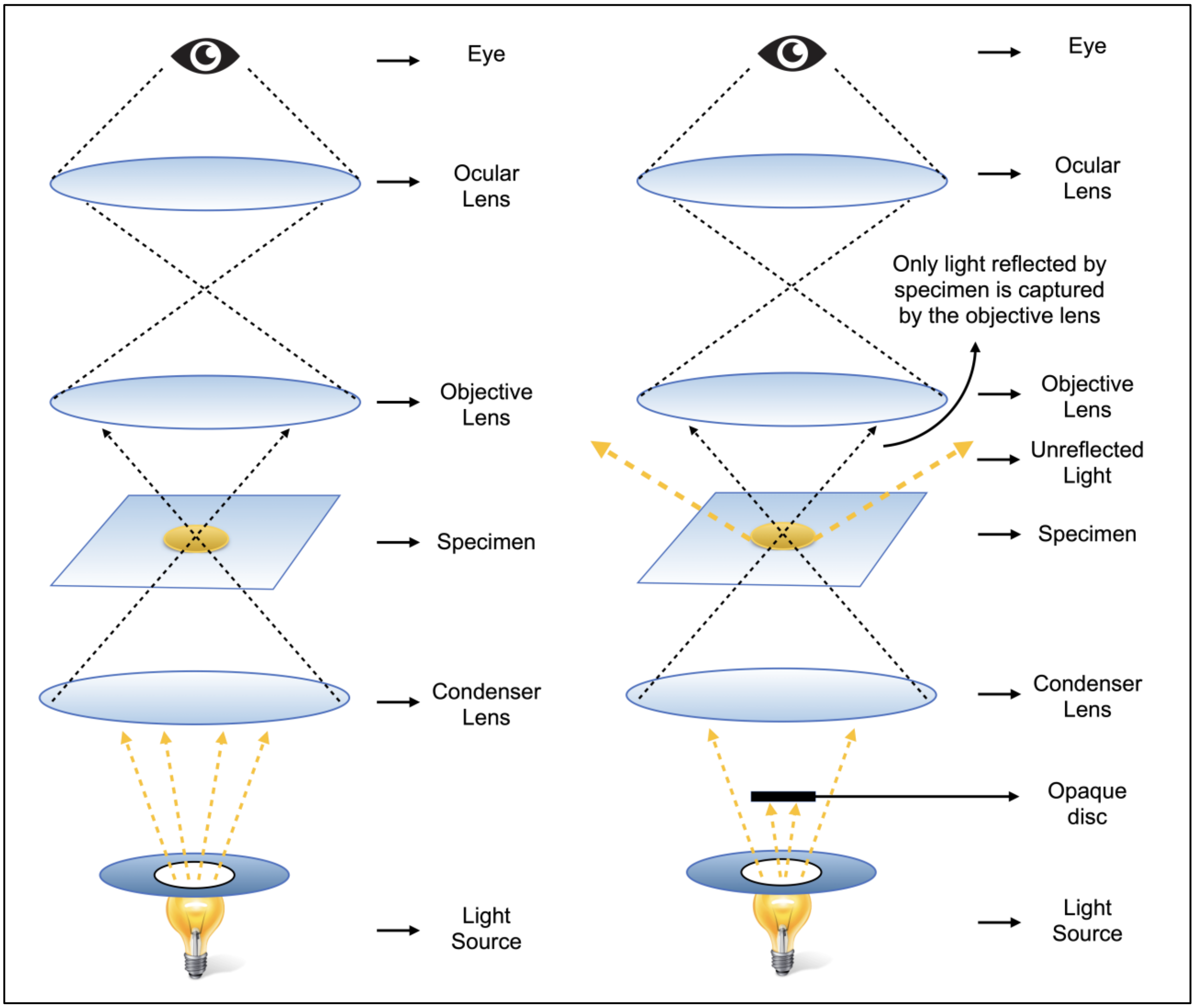


Figure 8. The working mechanism of a bright and dark-field microscope.

from around 20 to 80 nm(Pasquina-Lemonche, 2020). This cell wall is mainly made up of peptidoglycan, commonly known as mucopeptide or murein. In the case of thick cell walls, teichoic acids, polysaccharides, and peptide glycolipids are covalently bonded to the peptidoglycan. In contrast, Gram-negative bacteria have a thin peptidoglycan coating that is typically 5 to 10 nm thick. In species like *E. coli*, the peptidoglycan is likely only a thick monolayer. Beyond the peptidoglycan layer, Gram-negative bacteria have an outer membrane structure around 7.5 to 10 nanometers thick. In most Gram-negative bacteria, this membrane structure is noncovalently anchored to lipoprotein molecules, specifically Braun's lipoprotein, which is then covalently bonded to the peptidoglycan. The lipopolysaccharides of the Gram-negative cell envelope comprise a component of the outer membrane structure's outer leaflet. Moreover, The organization and overall dimensions of the outermost membrane of the Gram-negative cell envelope are similar to those of the

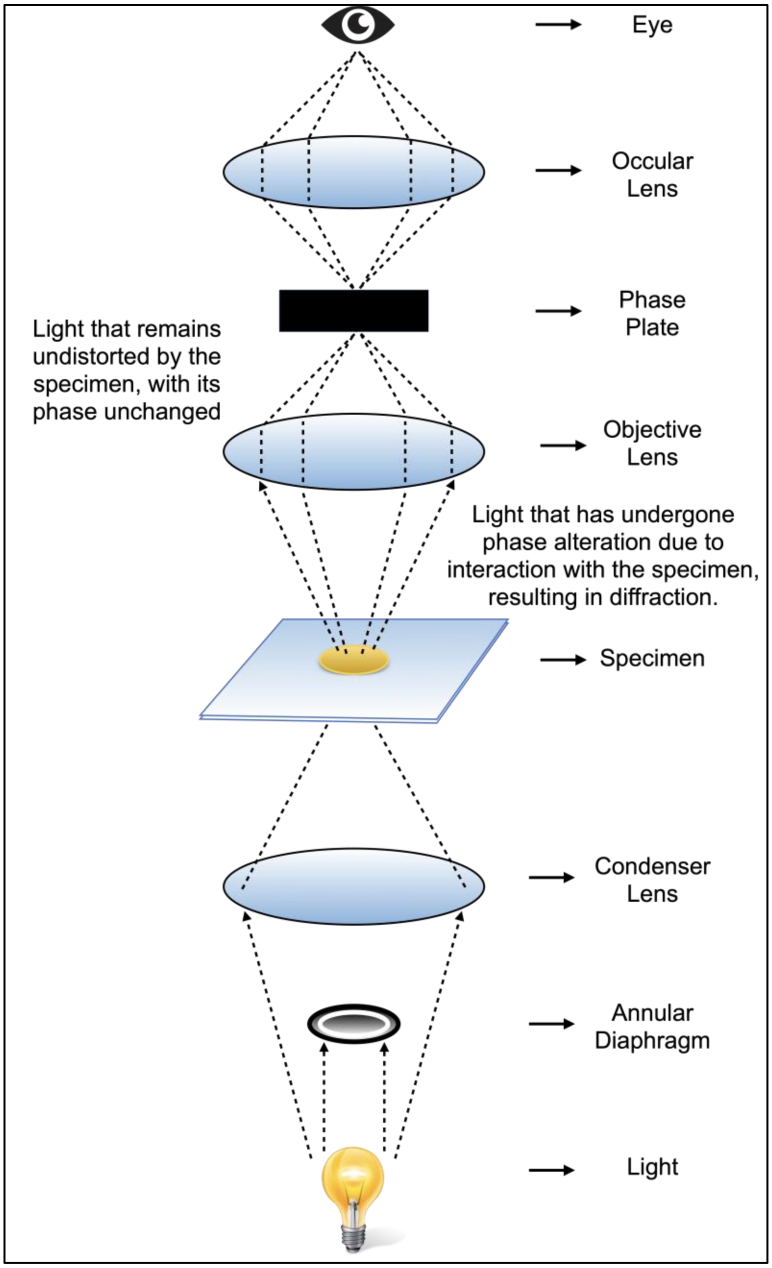


Figure 9. The working mechanism of a phase contrast microscope

plasma membrane, with a thickness of around 7.5 nm(Manon T., 2022).

3. Exploring Bacterial Morphology: Bridging Traditional Insights with the Latest Microscopic Advancements

3.1 Preparation of Sample Prior to the Examination

To prepare the samples (as depicted in Figure 6), bacteria are extracted and transferred onto a glass slide using a secondary glass slide. This extracted bacteria on the secondary slide underwent a series of stages, starting with fixation, dehydration, and

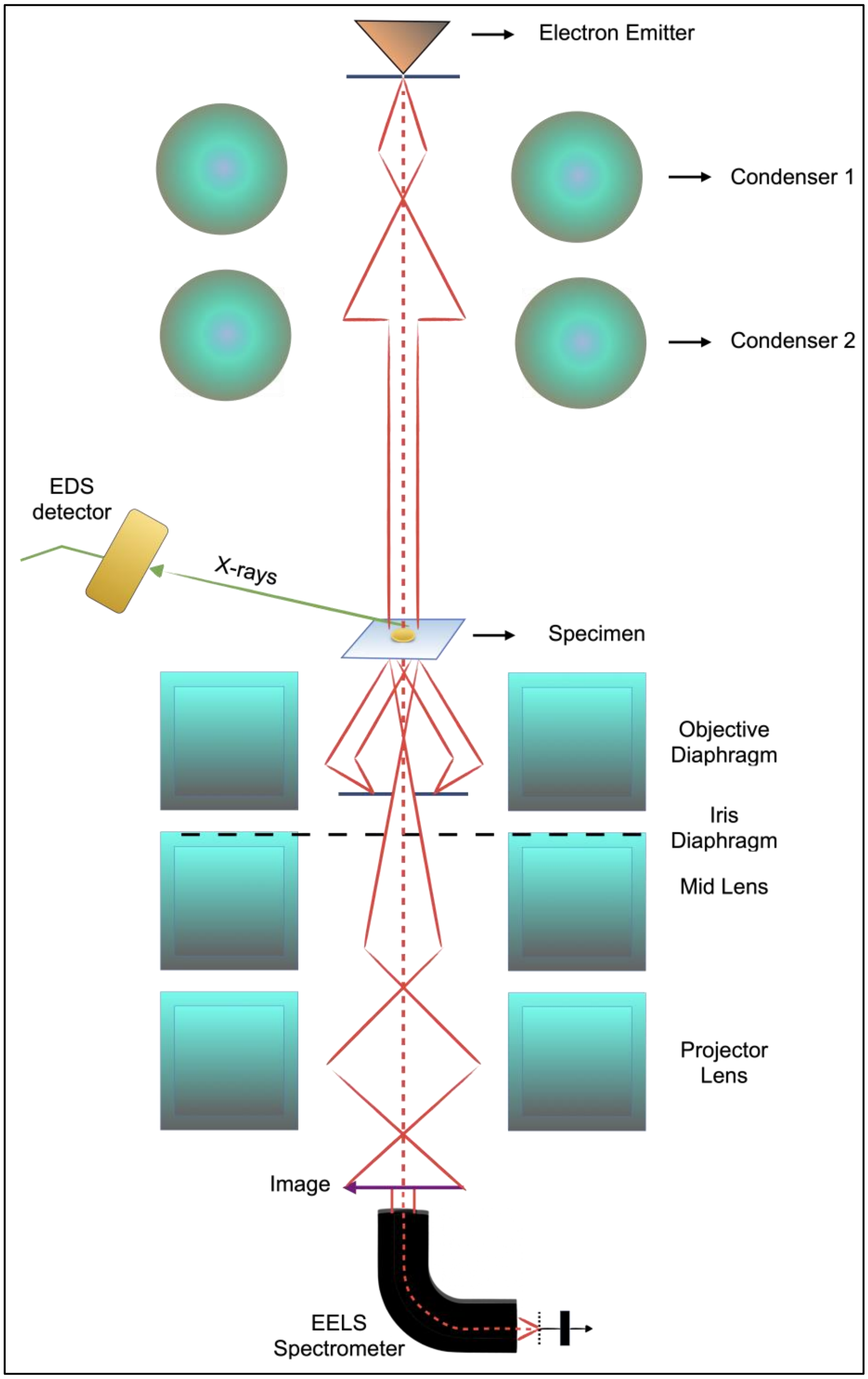


Figure 10. The working mechanism of a transmission electron microscopy (TEM).

lastly, air-drying(J. Sheikh et al., 2021). Initially, 4% glutaraldehyde is applied to the sample containing irradiated bacteria for the fixation process and left for 30 mins. Consequently, after 30 mins, the fixed bacteria undergo the next stage of

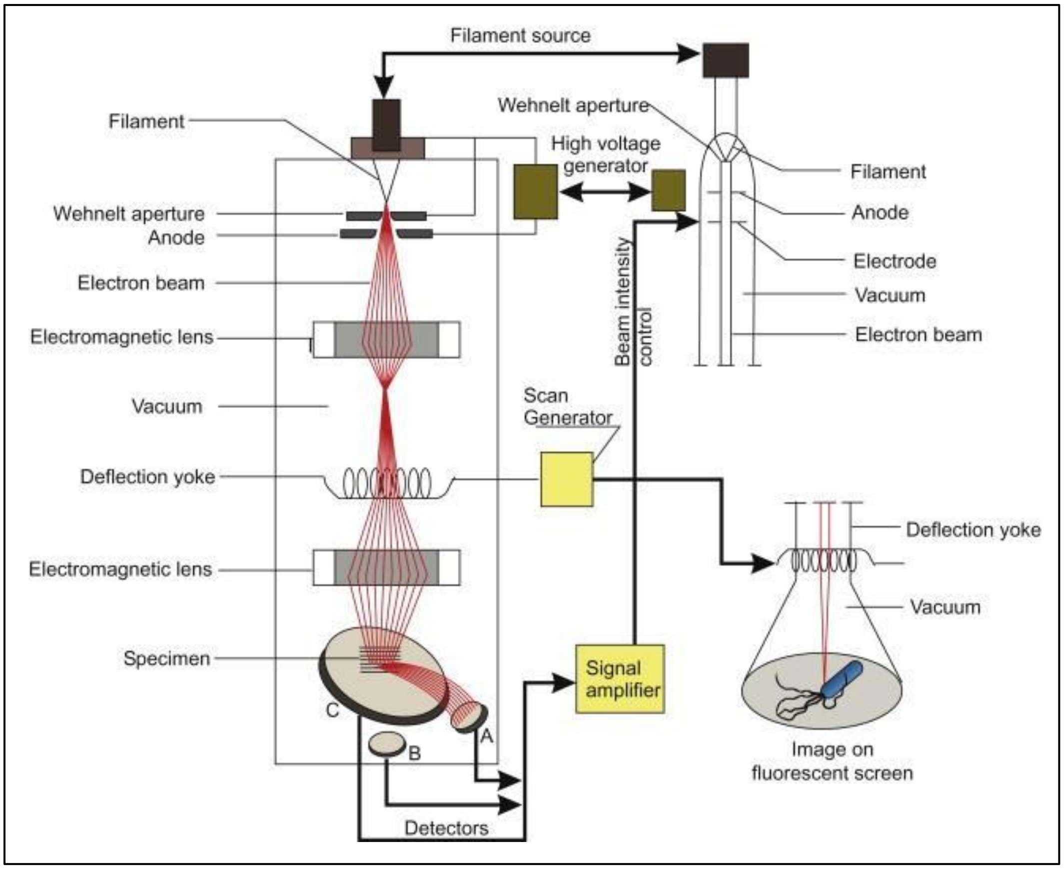


Figure 11. The working mechanism of a scanning electron microscope. Reprinted from Environmental Microbiology. 3rd Edition ed. Chapter 9 - Microscopic Techniques. 2015, Academic Press. (R., 2015)

dehydration by applying a series of ethanol concentrations (50%, 70%, 90%, and 100%) for 10 min each. The cover glass slides are then air-dried at ambient temperature before being sputter-coated. Finally, the readied samples can be observed using various imaging techniques, magnifications, and accelerating voltages. This approach enables observation and comparison of bacterial morphology before and after the different types of treatment.

4. Exploring the Microcosm: Bridging Traditions with Advanced Microscopy Techniques

4.1 Light Microscopy (LM)

The light microscope (Figure 7) plays a vital role in microbiology laboratories in helping to study and identify microorganisms. Its origins can be traced back to Van Leeuwenhoek's groundbreaking work(Lane N., 1677), which used a single rudimentary lens to visualize microorganisms for the first time. Light microscopy was essential in the discovery of the two main bacterial types, Gram-positive and Gram-negative, as established by Christian Gramme in the mid-1880s. Zernicke invented the phase-contrast microscope in 1932(Masters, 2020), which dramatically enhanced picture clarity, particularly when visualizing microorganisms. This approach is extensively used nowadays to image microorganisms without further staining. Bacteria are frequently classified via light microscopic examination based on their

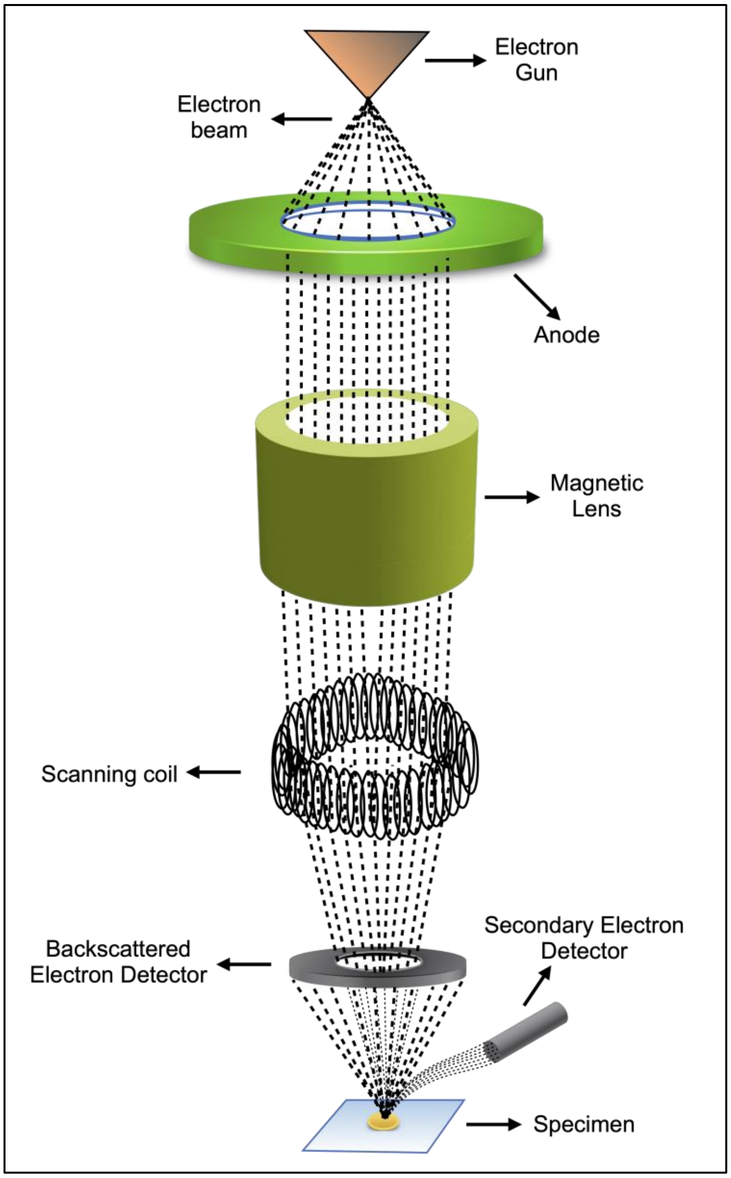


Figure 12. The working mechanism of a field-emission scanning electron microscope (Figure idea adopted from (C, 2022)

shape, size, and staining properties. While phase-contrast imaging allows for the use of unstained preparations, stained preparations usually provide better and clearer identification.

Bacteria can be distinguished by their shape, size, and staining properties, and the presence of distinctive features such as capsules, flagella, spores, or polyphosphate granules aids in identification. Simple staining processes are routinely used to emphasize the presence or lack of these structures, which helps with the overall identification process(Cui, 2022).

4.1.1 Bright- and Dark-Field Microscopy

Bright-field microscopy produces images by transmitting light through a specimen(Wang, 2023). As light passes through the specimen, part of it is absorbed, making it

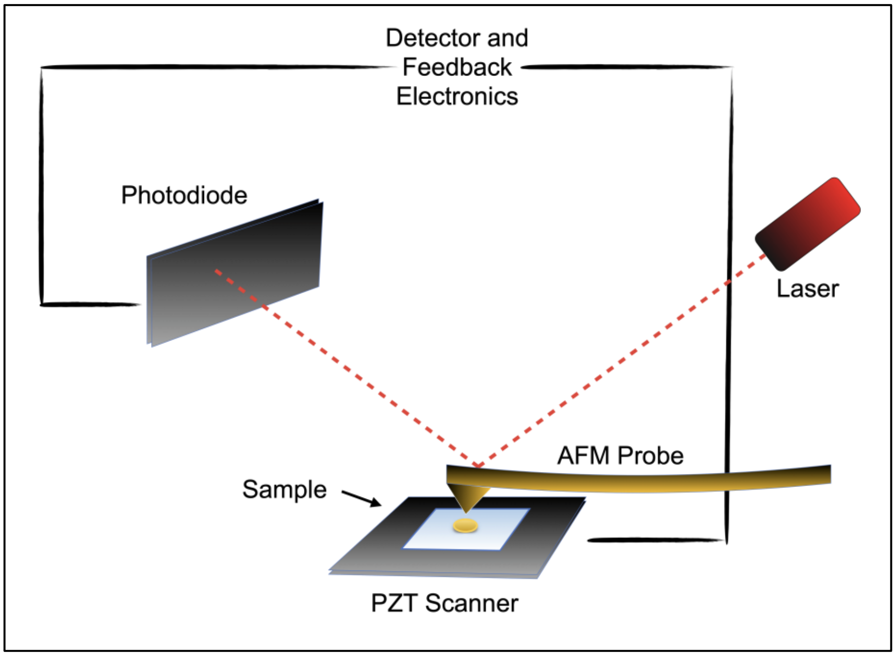


Figure 13: The working mechanism of an atomic force microscope.

look darker against the brightly lit background. This microscopy technique is widely used to examine the morphology of samples. However, due to the small size of microorganisms, particularly bacteria, bright-field microscopy frequently requires staining to increase contrast and attain the desired magnification(Parija, 2023), allowing for a sharper visualization of the structures of interest.

Dark-field microscopy, with contrast to bright-field (as depicted in Figure 8), improves contrast by selectively blocking some of the condenser's light(Shao, 2023), resulting in a dark background against which the dispersed light interacting with the specimen can be seen. This method is very beneficial for examining translucent specimens without the need for staining. Dark-field microscopy is useful for viewing live and unstained materials because it improves visibility and contrast for structures that would be difficult to see under bright-field settings. This technique selectively collects light scattered from the specimen's edges, producing a bright image against a dark background(Das, 2024). This approach is particularly useful for visualizing live specimens that have not been fixed or stained. For example, dark-field microscopy is frequently used to quantify the motility of bacteria and protozoa, offering a dynamic image of their movements. Additionally, it has been utilized to monitor the growth of bacterial microcolonies. Dark-field microscopy is a valuable tool for studying dynamic biological processes because it allows for the observation of unstained, living samples(Lei, 2024).

4.1.1.1 Limitations

Despite of advantages, compound light microscopes have a resolution of only 0.2 µm due to light's inherent physics. This theoretical constraint was achieved in the

Table 2. **Summary of the most recent developments in the application of these microscopic technologies as per recent literature**

Author	Technology Employment	Accelerating Voltage (kV)	Magnification	Microorganism	References
Xuesong H. *et al* (2024)	SEM (SU-70; Hitachi, Tokyo, Japan)	3.0	20.0k	*Brucella intermedia (B. intermedia)*	(Xuesong H., 2024)
Luyi S. *et al* (2024)	FESEM (S-4800; Hitachi, Tokyo, Japan)	10.0	20.0k	*Shigella flexneri* (*S. flexneri*)	(Luyi S., 2924)
Tao Z *et al* (2024)	SEM (EVO-LS10; Carl Zeiss AG, Germany)	10.0	5.0k	*Lactobacillus acidophilus* (*L. acidophilus*)	(Tao Z., 2024)
Jiahui L. *et al* (2024)	TEM (HT7800, Hitachi, Japan)	N/A	12.0k	*Aeromonas hydrophila* (*A. hydrophila*)	(Jiahui L., 2024)
Xiao Y. *et al* (2024)	SEM (SU8010, Hitachi Ltd., Japan) - TEM (cryo-TEM, Tecnai G2 F20, FEI, USA)	N/A	N/A	Methicillin-resistant *Staphylococcus aureus (MRSA)*	(Xiao Y., 2024)
Chen. *et al* (2024)	SEM (SU-8010; Hitachi Co., Tokyo, Japan) - TEM (FEI Co., Hillsboro, USA)	3.0, 200.0	10.0k, 9600.0k	*S. aureus*, *E. coli*	(X. Chen, et al, 2024)
Wenwen W. *et al* (2024)	SEM (JSM-IT300; JEOL)	N/A	N/A	*S. aureus,* *E. coli*	(Wenwen W., 2024)
Xiaotong Z. *et al* (2024)	FESEM (SEM, Quanta 250 FEG)	N/A	N/A	*S. aureus*	(Xiaotong Z., 2024)
Guangyao. *et al* (2024)	SEM (Tescan Vega3 and JSM-7610F Plus)	N/A	N/A	*S. aureus,* *E. coli*	(Guangyao W., 2024)
Anthony P. *et al* (2024)	SEM (Regulus 8100, Hitachi High-Technologies, Tokyo, Japan)	5.0	20.0k	*Pseudomonas lundensis* (*P. lundensis*), *Brochothrix thermosphacta* (*B. thermosphacta*)	(Anthony P. B., 2024)
Amrita J. *et al* (2024)	TEM (JEO, JEM-2100 HR, EELS, USA)	200.0	20.0k	*Serratia marcescens* (*S. marcescens*)	(Amrita J., 2024)
Somashree B. *et al* (2024)	AFM, FESEM, TEM	N/A	N/A	*S. aureus*	(Somashree B., 2024)
Maria E. *et al* (2024)	SEM (JEOL JSM 6010 PLUS/LA, Tokyo, Japan) - AFM (AIST-NT Smart SPM-1000, CA, USA)	20.0	5000.0	*Enterococcus faecalis* (*E. faecalis*)	(Maria, 2024)
Nada A. *et al*	FESEM	N/A	N/A	*S. aureus*	(Nada, 2024)

(2024)	(FEI Quanta FEG 250 SEM)				
Xiaoye *et al* (2024)	SEM (SEM Hitachi S-4800-II, Japan) - TEM (Hitachi HT7700, Japan)	N/A	N/A	*Pannonibacter phragmitetus* (*P. phragmitetus*)	(Xiaoye, 2024)
Ria D. *et al* (2024)	SEM (JEOL; JSM-6510 LA)	20.0	10.0	*E. coli*	(Ria, 2024)
Stefania *et al* (2024)	SEM (Supra 4000; Carl Zeiss, Germany)	1.0	10.0k	*P. aeruginosa*	(Stefania, 2024)
Yu C. *et al* (2024)	SEM (SEM, Quanta FEG 250, Japan)	5.0	8.0k	*S. aureus,* *E. coli*	(Yu, 2024)
Mohamed. *et al* (2024)	TEM (JEM 2100 LaB6, JEOL, Japan)	200.0	N/A	*S. aureus,* *E. coli*	(Mohamed, 2024)
Baoyan G. *et al* (2024)	SEM (Verios 460, FEI, Thermo Fisher, USA)	10.0	20.0k	*S. aureus,* *S. subtilis,* *L. monocytogenes*	(Baoyan. G., 2024)
Zhang *et al* (2024)	SEM (S-4800, Hitachi, Japan)	N/A	N/A	*S. aureus,* *E. coli*	(B. Zhang, 2024)
Xuan *et al* (2024)	SEM (SEM; Thermo scientific ApreoS LoVac, USA)	15.0	N/A	*S. aureus,* *E. coli*	(Xuan, 2024)
Zhang *et al* (2024)	TEM (H-7650, Hitachi, Tokyo, Japan)	N/A	20.0k, 12.0k	*S. aureus*	(X. Zhang, 2024)
Ma *et al* (2024)	FESEM (S-4800, Hitachi, Japan)	10.0	18.0k	*S. aureus,* *E. coli*	(Ma, 2024)
Iqbal T *et al* (2024)	SEM	N/A	30.0k	*E. coli*	(Iqbal, 2024)
Shaaban *et al* (2023)	SEM (JEOL JSM- IT200, Japan)	N/A	10.0k	*S. aureus,* *P. aeruginosa*	(Shaaban, 2023)
Zhang *et al* (2023)	SEM (Regulus 8100, Japan electronics Co. Ltd., Tokyo, Japan)	5.0	40.0k	*S. aureus*	(Zhang, 2023)
Zhi-Qiang *et al* (2023)	FESEM (SU8010, Hitachi High-Tech Co., Ltd, Shanghai, China)	3.0	20.0k	*Enibacillus polymyxa* (*E. polymyxa*)	(Zhi-Qiang, 2023)
Hamida *et al* (2023)	SEM (Zeiss Sigma 300 VP, GEMINI, Germany)	5.8	24.98k	*S. aureus*	(Hamida, 2023)
Cheng *et al* (2023)	SEM (SEM, Japan NTC JSM-6390LV) - AFM	N/A	15k	*E. coli*	(CHEN X-G., 2023)

Borislava ***et al*** **(2023)**	SEM (LYRA I XMU, Tescan Ltd., Czech Republic)	10.0	10.0k	*Blood microbiome* (*B. microbiome*)	(Borislava, 2023)
Hayat et al **(2023)**	SEM (JEOL JSM 6360LA, Akishima City, Tokyo, Japan)	30.0	20.0k	*S. aureus*	(Hayat, 2023)
Shehabeldine ***et al*** **(2023)**	TEM (JEOL-2010)	80.0	64k	*E. coli*	(Shehabeldine, 2023)
Arunachalam ***et al*** **(2023)**	SEM (SEM MX 63 and MX63L Olympus)	20.0	20.0k	*Naringi crenulate* (*N. crenulate*)	(Arunachalam, 2023)
Paramita ***et al*** **(2023)**	Light Microscope	N/A	N/A	*E. coli*	(Paramita, 2023)
Zhang ***et al*** **(2023)**	SEM (Regulus 8100, Hitachi, Japan)	N/A	N/A	*E. coli*	(Zhong, 2023)
Dorin ***et al*** **(2023)**	SEM (Carl-Zeiss-Strasse 22, Oberkochen, Germany)	20.0k	48.0k	*E. coli*	(Dorin, 2023)
Peiyuan ***et al*** **(2023)**	SEM (JSM-6360LV; JEOL, Japan)	10.0	25.0k	*Bacillus aryabhattai* (*B. aryabhattai*)	(Peiyuan, 2023)
Ribeiro ***et al*** **(2023)**	AFM (FlexAFM-NanoSurf AG, Liestal, Switzerland)	N/A	N/A	*E. faecalis*	(Ribeiro, 2023)
Luyao ***et al*** **(2022)**	Phase Contrast Microscopy (Olympus LUCPlan FL N)	N/A	N/A	*E. coli*	(Luyao, 2022)
Kahli ***et al*** **(2022)**	AFM (Olympus IX71)	N/A	N/A	*Pseudomonas fluorescens* (*P. fluorescens*)	(Kahli H., 2022)
Mitsuko ***et al*** **(2022)**	TEM (JEOL, JEM-2100 HC)	80.0	N/A	*E. coli*	(2022)

early 1930s, and despite continuous scientific curiosity, it hampered research into microscopic features within eukaryotic cells. Scientists indicated a wish to investigate the delicate inner morphology of cells, such as mitochondria or the nucleus, as well as to visualize the dynamic movement of bacterium flagella. This constraint led to the development of alternate microscopy techniques with higher-resolution capabilities(Manfred R., 2011). One significant disadvantage is its susceptibility to contaminants. Specimen slides must be meticulously cleaned to remove dust and grime, which might distort the images obtained. To ensure great image quality, specimens must be free of dust and air bubbles. Another constraint is the thickness of the specimens. Dark-field microscopy is best successful with thin specimens, whereas dense samples might reduce contrast and picture accuracy. As a result, not all samples are appropriate for dark-field imaging, and researchers must consider the properties of their specimens to acquire the best findings. These limitations highlight the significance of proper preparation and consideration when employing dark-field microscopy for specimen observation.

4.2 Phase-Contrast Microscopy (PCM)

Phase-contrast microscopy uses a series of diaphragms to distinguish and rejoin direct and diffracted light (as depicted in Figure 9) (Jixin, 2024). Köhler lighting focuses the light output onto a single focal plane. Light rays that pass through the Köhler diaphragm are focused as a hollow cone onto the specimen(Hayasaki, 2017). A circular diaphragm or diffraction plate is found in the objective's back focus plane. This diffraction plate, additionally referred to as a phase plate, alters the phase of light beams as they enter. The amount of light retardation through the plate causes the specimen to brighten or darken. This approach enhances the contrast and visualization of transparent specimens, making it very useful in the study of living cells and other transparent materials(R., 2015). Phase-contrast microscopy is a technique used to improve contrast in specimens(Häggmark, 2024), particularly for capturing high-contrast images of translucent specimens such as living cells. This approach takes advantage of the fact that numerous interior cell components are transparent but have varying densities. These varied densities interact uniquely with light, creating contrast between internal cellular components and the surrounding medium. Phase-contrast microscopy uses density differences to observe and study the dynamic and complicated architecture within living cells, eliminating the need for staining or other contrast-enhancing procedures(Glancy, 2023).

4.2.1 Limitations

Nonetheless, the approaches' wider acceptance was hampered by a number of problems associated with phase plate manufacture and charging(Nagayama K., 2008). This approach further improves contrast however lowers resolution by producing artefacts in structural details. Furthermore, the precision of quantitative data produced with phase contrast microscopes has some limitations, largely due to the presence of phase halo and shading-off effects(Kalampounias, 2024). These occurrences can contribute to uncertainties and errors in measurements, especially when analyzing refractive index variations across object borders(R. Hard., 2014).

4.3 Electron Microscopy (EM)

4.3.1 Transmission Electron Microscopy (TEM)

TEM is a technique for visualizing the internal structure of solids that uses a high-energy electron beam delivered through the material(Priyamvada, 2023). This configuration is similar to a standard optical microscope with transmission lighting, such as a biological microscope. TEM imaging along with spectroscopy approaches is based on the interaction of a high-energy electron beam (depicted in Figure 10), usually 80 keV or higher(Jia-Ye L, 2023). Figure 8 depicts how a beam from a suitable illumination system is directed onto the sample and then passed through it after being focused by electromagnetic lenses. The electrons that get transmitted and possibly diffracted after leaving the specimen to pass through further lenses, resulting in a picture of the specimen(Kandeeban, 2024). This allows for the analysis of sample shape and structure at nanometer scales. Furthermore, secondary signals generated by the specimen's interaction with the electron beam can reveal information on its composition at the nanoscale scale.

Over the last 10-15 years, two significant innovations have played critical roles in advancing and broadening the use of TE and its corresponding analytical approaches as powerful techniques for investigating the morphology, structure, and composition of Quantum Dots (QDs). The first breakthrough is the development of high-brilliance electron sources, often known as field emission guns (FEGs). Compared to traditional thermionic electron sources such as tungsten hairpins or pointed LaB6 rods, FEGs provide substantially greater electron beam levels, a substantially reduced minimum electron probe size (roughly one a nanometer), reduced electron-energy dispersion, elevated spatial and temporal coherence(CB., 2004; Reimer, 1997).

4.3.1.1 Limitations

TEM, while an effective method for high-resolution imaging, has significant drawbacks. High-resolution TEM (HR-TEM) images typically have a field of view of 100 nm^2(Yen-J. W., 2024), which limits their sampling capability. This constraint may impede the thorough study of larger structures or heterogeneous materials. Second, image interpretation is difficult since all TEM images and diffraction patterns are 2D projections of detailed 3D structures. This intrinsic constraint must be carefully considered when attempting to reproduce the complete spatial arrangement of specimens. Another important constraint is the possibility of electron beam damage, especially to light elements, biological samples, and soft materials(Kui, 2024). These materials' sensitivity to electron bombardment can cause structural changes and jeopardize the accuracy of studies. Furthermore, the requirement for a vacuum atmosphere in TEM settings limits the observation of functional materials in genuine "working" conditions, restricting TEM's application to real-world scenarios. Despite its impressive capabilities, these limits highlight the need for a cautious approach and supplementary methodologies in particular research contexts(C. Jia., 2010; L. Ruiz-Perez., 2021; O. Krivanek., 2014; R. B. G. Ravelli., 2020).

4.3.2 Scanning Electron Microscopy (SEM)

An image is formed with a SEM as an electron probe scans the specimen's surface (see Figure 10). This process generates a variety of signals, including secondary electrons, backscattered electrons, X-rays, Auger electrons, and photons of various energy(Manmohan. M., 2023). In general, the SEM uses these signals to capture the three-dimensional surface properties of specimens(Yancheng, 2024). The SEM has various benefits, including a large depth of focus, the capacity to study bulk samples at low magnification, and the ability to generate lifelike images. An electron gun and multiple condenser lenses collaborate in order to produce an electron beam (4 nm)(Xu, 2021). The rays of this beam are aligned using electromagnetic scan coils. Electron-accelerating voltages in the gun typically vary between 60 and 100 kV (kilovolts). The illumination source inside the gun is a tungsten filament heated to around 2700K. The heat causes electrons to be emitted from the filament tip. The surface topographical image of the specimen is generated by electrons that either reflect (backscatter) or emit (secondary electrons). To boost contrast in the SEM, coat the sample with a small layer of a conductive metal such as gold, palladium, or carbon(Tovlahanova, 2021). The image is formed by rastering the electron back and forth across the specimen surface.

4.3.2.1 Limitations

SEM comes with certain downsides, particularly with its size and expense. SEM is an expensive imaging tool due to the high initial and recurring costs. Furthermore, sample preparation for SEM analysis can create artefacts(Benjamin, 2024), thereby altering the precision of results. Another significant constraint is that SEM is restricted to solid, biological and inorganic materials that are small enough to fit within a vacuum chamber capable of tolerating modest vacuum pressure. This constraint limits the extent of SEM applicability by restricting the inspection of larger or non-solid specimens, reducing its adaptability in particular research situations(2016).

4.3.3 Field-Emission Scanning Electron Microscopy (FESEM)

In 1942, Zworykin introduced the first authentic SEM, demonstrating that topographic contrast could be achieved by constructively biasing the collector in relation to the specimen and obtaining a resolution of 50 nm employing an electron multiplier tube amplifying secondary electron emission. The field emission method, which uses a field emitter gun (FEG) to emit electrons, is the only electron source available for high-resolution imaging over a wide range of materials(El-Gomati, 2021). The SEM with FEG as the emitter is known as a FESEM(Y. Zhang, et al, 2024), which distinguishes it from a standard SEM. FESEM is an advanced microscopy tool with higher magnification that allows for the examination of minute details at lower voltages than regular SEMs in research settings.

FESEM is used to visualize fine topographic characteristics on the surfaces of complete or fragmented objects (as depicted in Figure 12). Scientists in biology, chemistry, and physics use this technology to detect objects as small as 1 nm. Applications include investigating cellular organelles, DNA materials, synthetic polymers, and microprocessor coatings. The FESEM further features a brighter electron source and a narrower beam size than most SEMs(Lewczuk, 2021), allowing magnifications of up to 500,000x. Another advantage is the capacity to obtain high-resolution imaging at very low accelerating voltages, which allows for the detection of fragile surface characteristics, electron-beam-sensitive materials, and non-conductive substances. FESEM sample criteria demand the use of dry, non-magnetic specimens(J Schilling., 2005). Powders, metals, and thin films are all acceptable analytical samples. Before analysis, users are directed to dissolve powder samples with particles larger than 100 nm in a suitable solvent and then deposit the solution onto a conducting substrate, such as copper, aluminum foil, or silicon, using drop casting or spin coating. Communication with the operator is recommended to go over these steps. Biological and liquid samples must be fixed and placed as coatings or drop-casted onto 1 cm conducting substrates(J. Sheikh et al., 2021), with full drying prior to the specified slot timings(Ganguly S., 1989; Oately, 1965; Singh KK., 1993). In addition, a sample quantity of 10 mg is required for analysis. Following these rules assures proper preparation and compatibility with the FESEM.

4.3.3.1 Limitations

FESEM normally requires a high vacuum (Ping-Yen. H., 2024) because gas molecules tend to disrupt the electron beam and the produced secondary and backscattered electrons required for imaging(Ramadhansyah P., 2020). Nonetheless, acquiring a large number of pictures in FESEM scans can significantly increase the time necessary for imaging, resulting in a significant rise in the overall cost involved with doing these scans(2016).

4.3.4 Atomic Force Microscopy (AFM)

The AFM is a type of scanning probe microscopy (SPM) that uses a small probe to traverse a surface rather than electrons or a light beam(Fukuda, 2024), as illustrated in Figure 13. This microscopy technique allows you to create three-dimensional maps of surfaces. Aside from AFM, additional types of SPMs include the scanning tunneling microscope (STM) and the near-field scanning optical microscope (NSOM). The AFM has a versatile tip that may be adapted in a variety of ways to examine surface attributes. The AFM, a more advanced iteration of the STM, can photograph a wide spectrum of surfaces at the nanoscale(Fotiadis D, 2002). Over the last decade, AFM has developed as an effective tool for obtaining nanostructure details and investigating the biomechanical aspects of biological samples. This encompasses a diverse spectrum of items, such as biomolecules and cells. The AFM's capacity to give high-resolution imaging and exact force measurements has made major contributions to our understanding of biological things' complicated architecture and mechanical properties. This technical innovation has created new pathways for inquiry and investigation in domains such as biology and biophysics, allowing scientists to investigate the nanoscale complexities of biological systems with unparalleled detail and accuracy(Hörber JK, 2003; Pelling AE, 2004).

AFM stands out as a scanning probe microscope with exceptional resolution measured in fractions of Angstroms. In comparison to the traditional optical microscope, the AFM has a resolution that is more than 1000 times higher. A micro- and nanoscale cantilever lies at the heart of its design, terminating in a sharp tip (probe) constructed of silicon or silicon nitride. This fine tip is used to carefully scan the surface of objects at the nanoscale. In general, carbon nanotubes (CNTs) can be used to the cantilever tip to improve image resolution to the Angstrom level, resulting in an even sharper tip. This augmentation improves image resolution when examining surfaces. However, it is important to highlight that CNT-based AFM tips are extremely brittle, necessitating special precautions during both mounting and surface scanning procedures(Emmanuel A. B.). The contact mode is a popular method for measuring surface force that involves scanning the sample closely with the cantilever tip. However, this mode has limitations, including a higher wear rate and the possibility of tip failure during scanning owing to constant contact. In contrast, the noncontact mode in AFM positions the tip at a short distance (usually 5-15 nm) above the sample surface, reducing direct contact. Recognizing the limitations of contact mode and tapping mode. AFM has been developed to address a variety of issues related to continuous contact scanning. Tapping mode establishes intermittent contact between the cantilever tip and the sample surface, minimizing difficulties such as friction, tip interactions with rough surfaces, electrostatic forces, and adhesion (Joshua, 2023; P. Gupta, 2006) .

4.3.4.1 Limitation

The scanning speed of an AFM is a significant constraint. Unlike an SEM, which can scan in near real-time, an AFM normally takes several minutes to complete a single scan(Yinan, 2020). While SEM allows for quick imaging, it may result in lower image quality. AFM's slower scanning speed is owing to the thorough and exact nature of its scanning technique, in which the cantilever tip meticulously traverses the sample surface, making it ideal for high-resolution imaging but less efficient in terms of speed. When deciding between AFM and SEM for specific imaging objectives, researchers and operators must examine the tradeoff between scanning speed and image quality(Rateesh B., 2014).

4.3.5 Microscopic Advances for Pathogenesis and Antimicrobial Drug Discovery

Furthermore, super-resolution microscopy has emerged as a powerful tool for studying biological processes, providing exceptional resolution by exceeding the diffraction limit of typical imaging techniques. Among these methods, SIM stands out as an especially well-suited super-resolution technology for biological applications. Its features include low phototoxicity and great resolution, making it ideal for researching complex biological structures and dynamics(Demmerle, 2017). In addition, STORM, a powerful super-resolution microscopy technique, enables the visualization of molecular-scale structures with nanoscale detail. This technology uses the stochastic blinking of fluorescent labels to precisely pinpoint individual molecules, allowing for the reconstruction of high-resolution images(Xu J, 2017). These methods are preferred for examining various changes and structures in morphology following different treatments, offering a broader depth of field and three-dimensional aspects of samples.

Microscopic imaging can help in drug discovery by shedding light on disease progression and identifying relevant therapeutic approaches. New technologies and improvements to current approaches are tackling earlier technical restrictions that have limited the value of microscopic imaging. These advancements aim to improve spatial resolution, and tissue penetration, overcome physical access difficulties, and increase experimental throughput. Such advancements include the development of super-resolution microscopes, the incorporation of multiphoton techniques into intravital and fiber-optic microscopy, and the automation of microscopy and image analysis for high-content screening. Collectively, these developments are broadening the range of tests and disease models used in early drug discovery, and in some cases, allowing the development of new ones(Bullen, 2008).

4.3.5.1 Super-resolution microscopy (SRM)

Optical SRM techniques show promise as suitable complements to established structural biology methods because they efficiently solve two fundamental constraints. For starters, the use of fluorescent markers allows for molecular specificity and great contrast, making it possible to analyze individual structures meaningfully without averaging. Second, SRM offers the inherent ability to directly visualize dynamic structural changes in living cells, providing insights into real-time cellular processes. While SRM is limited to measuring the positions of fluorescent labels and cannot photograph the full protein structure, the label's size will eventually limit its resolution to about one nanometer. However, when paired with data from structural biology approaches that provide molecular resolution, SRM shows promise in providing critical information about the conformations of particular complexes and their dynamic alterations inside living cells(Sheng, 2022).

4.3.5.2 Intravital Microscopy (IVM)

IVM has enabled researchers to monitor a wide range of biological constructions within living animals, allowing for the long-term tracking of single-cell dynamics. Researchers have used IVM to visualize and analyze complex biological processes in a variety of fields, including vascular biology, immunology, stem cell biology, and oncology. IVM has also been useful in quantitatively assessing the organization, structure, and function of blood vessels. Notably, it has been used to detect irregular hyperpermeability and heterogeneous blood flow in tumor microvessels caused by an imbalance of pro- and anti-angiogenic factors. These abnormalities in tumor blood arteries impair the efficacy of radiation and chemotherapy while promoting the development of more aggressive and metastatic cancer cells(Choo YW, 2020).

IVM has been used to track the mobility and localization of stem cells, assisting in the understanding of their roles. Certain studies have used IVM to visualize hematopoietic stem cells within the bone marrow niche and analyze their interactions, which are critical for maintaining appropriate stem cell activity. For example, researchers have developed IVM-guided transplantation techniques to introduce hematopoietic stem cells into mice's bone marrow, allowing them to analyze the functional properties of individual stem cells(Turcotte R, 2017).

4.3.5.3 Fibre-optic Microscopy

Fiber-optic imaging is a cutting-edge approach for visualizing cells and tissues in vivo, particularly when a regular light microscope is not an option(Vincent, 2006). Initially, fiber-optic imaging methods depended on optical fibers as simple, effective, and adjustable light conduits.

Recent advances, however, have brought fibers capable of serving as filters (e.g., for creating or controlling specific types of light(Pendão C, 2022). Furthermore, the miniaturization of accompanying fiber-optic gear, such as microelectromechanical system scanners and microlenses, helps to reduce the total size or compactness of many in vivo imaging systems(Myaing, 2006).

4.4 Recent Advances in Microscopic Technologies: Illuminating Bacterial Worlds through Multifaceted Studies

Numerous researchers have employed a variety of microscopic techniques to examine different Recent advances in the study of bacterial morphology have revolutionized our knowledge of the biology of bacterial cells, especially in relation to the Gram-negative outer membrane. The outer membrane has long been thought of as a permeability barrier, but more recent research has shown that it plays a wider and more varied role in cellular survival and physiology. By utilizing developments in microfluidics and microscopy, scientists have been able to decipher the mechanical, rheological, and structural characteristics of the outer membrane at different length scales. Key chemical elements and their interactions that control the outer membrane's spatial organization, limited diffusivity, and stress-bearing ability have been clarified by experimental and computational research. A rigorous examination of the findings' consequences for cellular fitness and survival is warranted because they imply significant relationships between physiology and structure of cells(Sun, 2022). In one study, AFM was employed to interrogate the morphologically distinct species of *S. aureus* and *B. subtilis*. The investigation encompassed both live cells and purified peptidoglycan samples. The findings revealed intricate details of the bacterial cell envelope architecture. Upon examination, it was observed spectral, spatial, and polarisation filters), as well as beam-splitters and nonlinear devices microorganism-related phenomena. AFM, TEM, SEM, and FESEM are some of the most widely used techniques utilized in contemporary study. Table 2 delivers a comprehensive overview of the cutting-edge applications employing microscopic technologies, sourced from an in-depth and meticulous review of the current scholarly literature. This compilation showcases cutting-edge advancements and their significance in a wide range of scientific investigations.

4.5 Integrating Recent Breakthroughs in Bacterial Morphology Research

that the mature surface of live cells exhibited a landscape characterized by large pores, reaching diameters of up to 60 nm and depths of up to 23 nm. Moreover, these findings offered valuable insights complementary to traditional structural biology approaches, providing a deeper understanding of bacterial morphology and physiology.

Utilizing cutting-edge microscopy methods like FESEM to acquire a profound understanding of the structure and morphology of the recently synthesized magnetic carbon nanofibers (MCF) was another innovation in one recently conducted study(Pimchanok, 2022). With the aid of these methods, scientists were able to examine the complex network and pore structure of MCF in three dimensions, which are essential for figuring out how well it can absorb contaminants. Furthermore, the distribution and homogeneity of nanoparticles within the material could be observed by microscopy, providing valuable information for optimizing the synthesis procedure and raising the adsorbent's efficacy. The relationship between MCF's structure and its ability to remove pollutants was holistically understood thanks to the integration of microscopy, which made it possible to develop and use MCF in water purification procedures in an optimized manner.

Our knowledge of the shape and function of bacterial cells has been completely transformed by recent advances in the field of bacterial morphology study. The fine intricacies of bacterial morphology have been revealed at previously unheard-of resolutions by researchers thanks to developments in imaging technologies, computational modeling, and biochemical analysis. These discoveries have added to our understanding of cellular architecture and clarified the physiological importance of certain morphological characteristics.

5. Conclusion and Perspectives

Biofilms play an important role in antimicrobial resistance because they provide a safe environment for microorganisms, insulating them from antimicrobial agents and the host immune response. Researchers can observe biofilms in real-time and in great detail using modern microscopy techniques such as super-resolution microscopy, confocal microscopy, and electron microscopy. This enables them to study the formation, structure, and dynamics of biofilms in a variety of settings, including medical devices, implants, and natural surfaces. With this in-depth understanding, scientists can create targeted interventions to prevent the growth of biofilms and improve the efficiency of antibiotic therapies. For instance, they can create nanoparticles that pierce biofilms to deliver antimicrobial drugs directly to microbial cells or create antimicrobial coatings for medical equipment that inhibit biofilm attachment. Furthermore, the study of microbial behavior in biofilms, especially the mechanisms behind antibiotic resistance, is made easier by microscopy. By observing the microscopic interactions between microbes and antimicrobial agents, researchers can discern resistance mechanisms, including but not limited to enzymatic degradation, cell membrane permeability alterations, and efflux pumps. Microscopical technologies enable the development of novel approaches to counteract antimicrobial resistance by elucidating the intricate interactions between biofilms and antimicrobial agents. These tactics could involve combination treatments that focus on several resistance routes, individualized treatment plans based on unique biofilm properties, or innovative antimicrobial drugs that prevent the production of biofilms without causing resistance.

The field of optical-based bacterial morphology studies has grown significantly in recent decades. This article provides a thorough examination of common bacterial morphologies, diving into their numerous kinds, and highlights current advances in traditional microscopic techniques in this subject. Despite of numerous availability of microscopic technologies, most of the recent studies employed SEM(Borislava, 2023; Dorin, 2023; Peiyuan, 2023; Zhang, 2023), FESEM (Ma, 2024; Nada, 2024; Somashree B., 2024; Xiaotong Z., 2024; Zhi-Qiang, 2023) and TEM (Amrita J., 2024; Mitsuko, 2022; Mohamed, 2024; Shehabeldine, 2023; Xiaoye, 2024) for the bacterial morphological examination, underscoring the significance of these high-resolution imaging techniques for bacterial morphological examination. Despite the availability of a wide range of other microscopic technologies, recent studies have shown that SEM, FESEM, and TEM are the most commonly used methods for thoroughly examining bacterial morphology, however; the financial limitations pose a major challenge for scientists in accessing and utilizing these advanced microscopic tools(2016). These high-resolution imaging techniques permitted researchers to get deep insights into the structural properties of bacteria, which reflects their popularity in current scientific investigations. Other technologies, such as phase contrast microscopy, were used infrequently in the investigations(Paramita, 2023), compared to the dominating prevalence of the other microscopic technologies.

Recent studies (L. H. Chen et al., 2020; Papa R., 2020; Rutala, Gergen, & Weber, 2010; J. Sheikh, et al, 2024; J. Sheikh, et al 2023; J. Sheikh, Swee, T.T., Saidin, S. et al. , 2024) have been conducted to assess the efficacy of disinfection in the treatment of bacterial samples, but they have offered restricted information by neglecting the morphological aspects. As we move forward, expanding the range of technologies used for bacterial morphological investigations may provide complementary viewpoints and reveal intricacies in microbial structures. Integrating developing technology and approaches into future research projects may provide a more thorough understanding of bacterial morphology, bolstering advancements in the field. A collaborative strategy that includes a broader range of imaging techniques has the potential to improve our understanding of the complex world of bacterial structures and functions. This, in turn, could lead to novel applications in a variety of scientific and medical sectors, propelling the advancement of microbiological research in the years ahead.

Conflict of Interest

The authors declare that they have no competing interests.

Funding

This work was supported by the institutional funds provided to the corresponding authors.

Study Approval

NA

Consent Forms

NA.

Authors Contribution

JS and TTS carried out all the data collection, bench work, and manuscript writing. SS and CLS helped in data collection and statistical analysis. SAM conceptualized and supervised the study.

Data Availability

Data is available upon reasonable request from the corresponding author.

Acknowledgment

The corresponding author thanks all the coauthors and laboratory fellows for their help during this project.

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