Microbiology

Unit 1 · Foundations: Microbial World and Microscopy

Microbiology starts with two questions: what are microbes, and how do we see them? This unit tells how microbes were discovered and how they are sorted, named and identified, then teaches the microscopes and stains that every microbiology lab uses. After it you can compare prokaryotic and eukaryotic cells, classify and identify a microbe from test results, calculate what a microscope can resolve, and read a Gram stain or an acid-fast smear.

Taught at 8 of 10 | Usually Sem 3 | 9 chapters

About this unit

The unit has two halves. Chapters 1.1 to 1.5 are about the microbes themselves: how microbiology grew from hand-made lenses to genome sequencing, how prokaryotic and eukaryotic cells differ, what kinds of microbes exist, and how they are classified, named and identified by biochemical and molecular tests. Chapters 1.6 to 1.9 are about the tools: light microscopes (bright-field, dark-field, phase contrast), fluorescence and electron microscopes, and the simple, Gram and special stains that give cells colour. The two halves meet at the lab bench, where a stain and a microscope are the first step in identifying any microbe. Each group of microbes gets its own chapter later: bacteria, fungi, algae and protozoa in Unit 2, viruses in Unit 3.

Chapters

1. History, scope and branches of microbiology
2. Prokaryotic and eukaryotic cells compared
3. Microbial diversity and types of microorganisms ADVANCED
4. Classification, taxonomy and identification of microbes
5. Biochemical and molecular characterization of microbes
6. Light microscopy: bright field, dark field, phase contrast
7. Fluorescence and electron microscopy
8. Staining: simple, Gram and differential stains
9. Special stains: acid-fast, endospore, capsule, flagella

How to use this book

- Read the lead paragraph and the figure of each chapter first; together they carry the main idea.
- Cover the solution of each worked example and work it yourself on paper, with units, before you check.
- Answer every "Check yourself" question in your head or on paper before you open it.

1.1 History, scope and branches of microbiology

Microbiology is the study of living things too small to be seen clearly with the naked eye: bacteria, archaea, fungi, algae and protozoa, together with the non-cellular agents (viruses, viroids and prions). Its history is the story of three questions being answered: do microbes exist, where do they come from, and what do they do? Microscopes answered the first, Pasteur's flasks the second, and the germ theory of disease and fermentation the third.

Seeing microbes for the first time

Robert Hooke's book *Micrographia* (1665) showed what a compound microscope could reveal; he used the word "cell" for the small boxes he saw in cork. The first person to see bacteria and protozoa was Antonie van Leeuwenhoek, a cloth merchant in Delft (the Netherlands), who ground single lenses that magnified a few hundred times. In 1676 he described moving "animalcules" in pepper water in a letter to the Royal Society of London, and he later drew bacteria scraped from his own teeth. He is called the father of microbiology.

The end of spontaneous generation

Spontaneous generation (abiogenesis) was the belief that life arises from non-living matter: maggots from meat, microbes from broth. Biogenesis is the opposite idea: living cells come only from living cells. The argument took two centuries to settle.

1. **Francesco Redi (1668)** kept meat in open jars and in jars covered with gauze. Maggots appeared only where flies could lay eggs. But microbes were too small for this test.
2. **John Needham (1740s)** boiled mutton broth briefly, sealed it and still saw microbes grow, which seemed to support spontaneous generation.
3. **Lazzaro Spallanzani (1760s)** boiled broth longer in flasks sealed by melting the glass; they stayed clear. Critics replied that sealing kept out a "vital force" carried by air.
4. **Louis Pasteur (1861)** answered them with swan-neck flasks that let air in but trapped dust in the bend of the neck (see the worked example).

5. **John Tyndall (1870s)** found that hay infusions needed repeated heating, and Ferdinand Cohn showed why: some bacteria form heat-resistant endospores. Sterilization methods are in Unit 5, *Physical control: heat, filtration and radiation*.

Germ theory and the golden age (1857–1914)

Pasteur's studies of wine and beer showed that fermentation is done by living microbes: yeasts make alcohol, and contaminating bacteria turn wine sour. Gentle heating to kill the spoilers is still called pasteurization. If microbes cause fermentation, specific microbes might cause specific diseases: this is the germ theory. Ignaz Semmelweis (1847) cut deaths from childbed fever in a Vienna hospital by making doctors wash their hands in chlorinated lime, and Joseph Lister (1867) began antiseptic surgery with carbolic acid (phenol). Pasteur went on to vaccines against anthrax (1881) and rabies (1885), building on Edward Jenner's use of cowpox against smallpox (1796).

Robert Koch supplied the proof. In 1876 he showed that *Bacillus anthracis* causes anthrax. He grew pure cultures on solid media: first gelatin, then agar (suggested by Fanny Hesse) in the dish designed by Julius Petri (1887). He found the tuberculosis bacillus (1882) and isolated the cholera vibrio in Calcutta (1884). His reasoning is known as Koch's postulates:

1. The same microbe is present in every case of the disease and absent from healthy hosts.
2. It is isolated from the sick host and grown in pure culture.
3. The pure culture causes the same disease when inoculated into a healthy, susceptible host.
4. The same microbe is isolated again from that experimentally infected host.

The postulates fail for healthy carriers (people who shed *Salmonella* Typhi without illness), for microbes that will not grow in the lab (*Mycobacterium leprae*, *Treponema pallidum*), for diseases with no animal model, and for diseases caused by several microbes together.

India is part of this story. Ronald Ross, working in Secunderabad in 1897, showed that mosquitoes carry the malaria parasite (Nobel Prize, 1902). Waldemar Haffkine made vaccines against cholera (1892) and plague (1897); his Bombay laboratory became today's Haffkine Institute. In Kolkata, Sambhu Nath De showed in 1959 that *Vibrio cholerae* causes diarrhoea through a toxin.

After 1890 the field branched out. Dmitri Ivanovsky (1892) and Martinus Beijerinck (1898) found that tobacco mosaic disease passes through bacteria-proof filters, which began virology; Beijerinck and Sergei Winogradsky founded microbial ecology. Paul Ehrlich's Salvarsan (1910) began chemotherapy and Alexander Fleming's penicillin (1928) the antibiotic era. Later came Carl Woese's discovery of the archaea (1977) and the PCR (1983), now run with a heat-stable DNA polymerase from the hot-spring bacterium *Thermus aquaticus*.

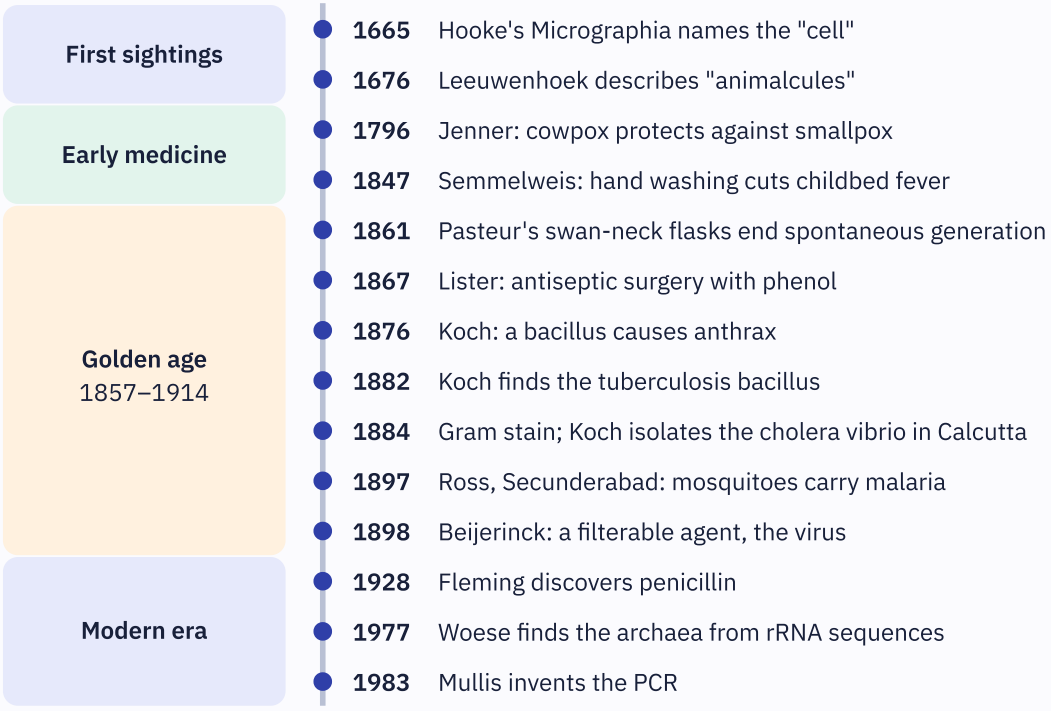


Figure 1.1.1 Fourteen landmarks of microbiology, grouped into four eras (rows are evenly spaced, not to scale). Notice how crowded the golden age is: in about forty years microbes were linked to fermentation, surgery, anthrax, tuberculosis, cholera and malaria.

Scope and branches

Some branches are named after the organisms studied: bacteriology, virology, mycology (fungi), phycology (algae), protozoology and parasitology. Others are named after what the knowledge is used for:

BRANCH	WHAT IT COVERS	EXAMPLE
Medical	Disease-causing microbes, diagnosis, vaccines	TB sputum smear
Food and dairy	Fermented foods, spoilage, food safety	Curd, idli batter
Industrial	Products made by microbes at large scale	Penicillin, citric acid, ethanol
Agricultural and soil	Soil fertility, plant disease, biofertilizers	*Rhizobium* in pulse root nodules
Environmental and aquatic	Water quality, sewage, clean-up of pollution	Sewage treatment plant
Genetics and biotechnology	Microbial genes and their use as tools	Human insulin made in *E. coli*

So the objectives of microbiology are to understand microbes, to control the harmful ones (disease and spoilage), to use the helpful ones (fermentation, antibiotics, enzymes, vaccines, biofertilizers), and to use simple microbes such as *E. coli* and yeast as models for all of biology.

WORKED EXAMPLE

Question (8 marks). Describe Pasteur's swan-neck flask experiment. Predict what happens to the boiled broth when (a) the neck is left intact, (b) the neck is broken off, (c) the flask is tilted so that broth runs into the bend and back. What did the experiment prove?

1. **Set-up.** Nutrient broth is put in a flask, the neck is heated and drawn into an S-shaped curve, and the broth is boiled to kill the microbes already in it. The neck stays open to the air, which answers the "vital force in air" objection.
2. **(a) Intact neck.** Air moves in and out, but dust particles carrying microbes settle on the moist walls of the bend. The broth stays clear for months.
3. **(b) Neck broken.** Dust falls straight into the broth, which turns cloudy (turbid) with growth within a day or two.
4. **(c) Flask tilted.** The broth washes over the trapped dust and carries microbes back down; it turns turbid.
5. **Conclusion.** Microbes in the broth come from microbes in the air, not from the broth itself. Air reached the broth in (a), so boiling had not destroyed any "vital force". This supports biogenesis.

Examiners look for a labelled flask sketch, the role of the bend, the broken-neck control and a one-line conclusion.

EXAM TRAPS

- Leeuwenhoek did not invent the microscope; he made better single lenses and was the first to see bacteria. Hooke saw cells in cork, not bacteria.
- Needham's result seemed to **support** spontaneous generation; Spallanzani's opposed it. Students often swap the two.
- Pasteur showed that microbes cause fermentation; Koch's postulates proved that a specific microbe causes a specific disease.
- Write all four postulates in order. The favourite follow-up is "state the limitations": give carriers and *M. leprae* as examples.

CHECK YOURSELF

– REMEMBER **Who first described bacteria, when, and with what kind of microscope?** 2 marks

Antonie van Leeuwenhoek, in 1676, with a hand-made single-lens microscope magnifying a few hundred times.

– REMEMBER **State Koch's postulates.** 4 marks

Microbe present in every case and absent in healthy hosts; isolated and grown in pure culture; the pure culture causes the same disease in a healthy susceptible host; the same microbe is re-isolated from that host.

– UNDERSTAND **Why did Spallanzani's sealed flasks fail to convince his critics, and how did Pasteur's design answer them?** 4 marks

Sealing kept out air, so critics said a vital force in air was missing. Pasteur's swan neck let air in freely but trapped dust and microbes in the bend, and the broth still stayed sterile.

– APPLY **A hay infusion boiled once for 15 minutes turned cloudy; one boiled for a few minutes on three days in a row stayed clear. Explain.** 4 marks

Hay carries bacterial endospores that survive one boiling. Between heatings the spores germinate into vegetative cells, which the next boiling kills. This intermittent heating is tyndallization.

– ANALYZE ***Mycobacterium leprae* has never been grown on lab media. Which postulates cannot be met, and how is it still accepted as the cause of leprosy?** 4 marks

Postulates 2 and 3 need a pure culture, so they fail. The cause is accepted because acid-fast bacilli are found in the lesions of every case, the bacterium multiplies in mouse footpads and armadillos, and its DNA is found in patients' tissue.

BEYOND THE SYLLABUS

Since the 1990s microbiology has become a genome science: the first complete bacterial genome, of *Haemophilus influenzae*, was published in 1995. CRISPR–Cas9, a bacterial defence against viruses, became a gene-editing tool and won the 2020 Nobel Prize in Chemistry. Search "One Health antimicrobial resistance" to read further; none of this is needed for the exam.

1.2 Prokaryotic and eukaryotic cells compared

Every cellular microbe is built on one of two plans. A prokaryotic cell (Greek *pro*, before; *karyon*, kernel or nucleus) has no nucleus: its DNA lies in the cytoplasm. Bacteria and archaea are prokaryotes. A eukaryotic cell (*eu*, true) keeps its DNA inside a membrane-bound nucleus and has other membrane-bound organelles; fungi, algae and protozoa are eukaryotes, like plants and animals. The two plans also differ in size, ribosomes, walls, membranes and division, and many antibiotics work because of these differences.

The prokaryotic plan

A typical bacterium such as *Escherichia coli* is a rod about 1 µm wide and 2 µm long (1 µm, one micrometre, is 10^{-6} m). From the outside in, it has an optional capsule, a cell wall (made of peptidoglycan in bacteria), a plasma membrane and the cytoplasm. Respiration happens on the plasma membrane, because there are no mitochondria, and photosynthetic bacteria have no chloroplasts. The DNA is usually one circular chromosome packed into a region called the nucleoid, with no membrane around it. Small extra DNA circles, plasmids, often carry genes for antibiotic resistance. Ribosomes are 70S. Flagella, when present, rotate like propellers. The cell divides by binary fission, and because no membrane separates DNA from ribosomes, an mRNA is translated while it is still being made. Unit 2 (*Bacterial morphology and cell structure*) covers each of these parts.

The eukaryotic plan

Eukaryotic cells are usually 10–100 µm across, although a yeast cell is only about 5 µm. The nucleus is bounded by a double membrane, the nuclear envelope, with pores; inside are several linear chromosomes wound on histone proteins, and a nucleolus where ribosomes are assembled. The cytoplasm holds 80S ribosomes, free or on the rough endoplasmic reticulum (ER), the Golgi apparatus, lysosomes and vacuoles, mitochondria for respiration and, in algae, chloroplasts. A cytoskeleton of actin filaments and microtubules lets the cell change shape, move material inside it and swallow food by phagocytosis, as an amoeba does. Eukaryotic flagella and cilia have the "9 + 2" arrangement of microtubules and beat like whips. Membranes contain sterols: ergosterol in fungi, cholesterol in animals. Cells divide by mitosis, and many also reproduce sexually through meiosis.

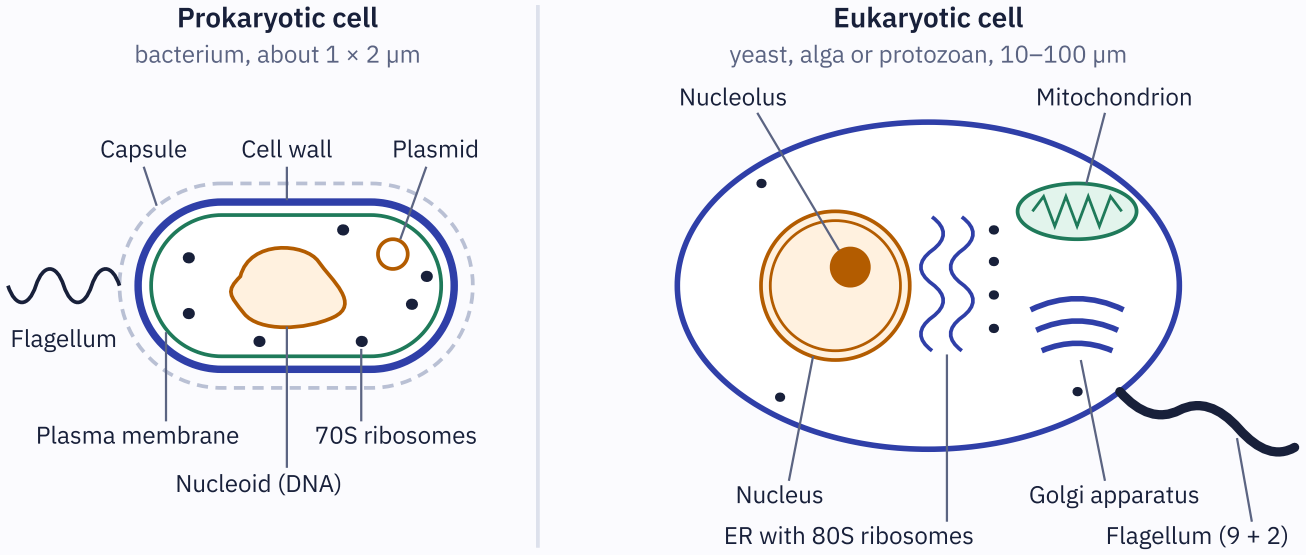


Figure 1.2.1 The two cell plans (not to scale: the eukaryotic cell is usually ten or more times wider). Notice that the prokaryote's DNA has no membrane around it, while the eukaryote's nucleus, mitochondrion, ER and Golgi are all bounded by membranes.

Side by side

FEATURE	PROKARYOTE	EUKARYOTE
Typical size	0.2–2 µm wide	10–100 µm
Nucleus	None; DNA in a nucleoid	Membrane-bound, with nucleolus
Chromosomes	Usually one, circular; no histones in bacteria	Several, linear, on histones
Ribosomes	70S (50S + 30S)	80S (60S + 40S); 70S inside mitochondria and chloroplasts
Membrane-bound organelles	Absent	Mitochondria, ER, Golgi, lysosomes, chloroplasts
Site of respiration	Plasma membrane	Mitochondria
Cell wall	Peptidoglycan in bacteria; absent in *Mycoplasma*	Chitin (fungi), cellulose (algae), none (protozoa)
Sterols in membrane	Absent (except *Mycoplasma*)	Present
Flagella	About 20 nm thick, rotate	About 200 nm thick, 9 + 2 microtubules, whip-like
Endocytosis, cytoplasmic streaming	Absent	Present

FEATURE	PROKARYOTE	EUKARYOTE
Division	Binary fission	Mitosis; meiosis in sexual reproduction
Gene expression	Transcription and translation coupled; introns rare	Separated by the nuclear envelope; introns common

Where archaea fit, and why the difference matters

Archaea have the prokaryotic plan but are a separate domain (Chapter 1.4). Their membrane lipids are joined by ether links (bacteria and eukaryotes use ester links), their walls never contain peptidoglycan, and many wrap their DNA on histone-like proteins. So "prokaryote" describes a cell plan, not one family tree.

Mitochondria and chloroplasts have their own circular DNA and 70S ribosomes, and they divide by fission. This supports the endosymbiont theory: they descend from bacteria that an early eukaryotic cell engulfed.

The differences also give selective toxicity, the basis of antimicrobial drugs (Unit 5, *Antibiotics and how they act*). Penicillin blocks peptidoglycan synthesis, which human cells do not need. Streptomycin and tetracycline act on 70S ribosomes and largely spare 80S ones. Amphotericin B binds ergosterol, so it damages fungal membranes more than human ones.

WORKED EXAMPLE

Question (6 marks). A coccus is a sphere 1 μm in diameter; a yeast-like eukaryotic cell is a sphere 10 μm in diameter. (a) Find the surface-area-to-volume ratio of each. (b) A nutrient molecule diffuses from the membrane to the centre. Using $t \approx x^2/(2D)$, where x is the distance and D the diffusion coefficient (take $D = 1 \times 10^{-10}$ m²/s, a round value for a small molecule in cytoplasm), estimate the time in each cell. (c) What do the answers say about cell size?

1. For a sphere of radius r, surface area $A = 4\pi r^2$ and volume $V = (4/3)\pi r^3$, so $A/V = 3/r$.
2. Coccus: $r = 0.5$ μm, so $A/V = 3 \div 0.5 = 6$ μm^{-1}. Eukaryotic cell: $r = 5$ μm, so $A/V = 0.6$ μm^{-1}. The coccus has 10 times more membrane per unit volume to take in food.
3. Coccus: $x = 0.5$ μm $= 5 \times 10^{-7}$ m, $x^2 = 2.5 \times 10^{-13}$ m², $t = 2.5 \times 10^{-13} \div (2 \times 10^{-10}) = 1.25 \times 10^{-3}$ s, about 1 ms.
4. Eukaryotic cell: $x = 5$ μm $= 5 \times 10^{-6}$ m, $x^2 = 2.5 \times 10^{-11}$ m², $t = 2.5 \times 10^{-11} \div (2 \times 10^{-10}) = 0.125$ s.
5. Time grows with the square of distance: 10 times the radius gives 100 times the time. A small prokaryote can rely on diffusion and grow fast; a large eukaryotic cell needs internal membranes, vesicle transport and a cytoskeleton to move material.

EXAM TRAPS

- 30S + 50S makes 70S, not 80S. The svedberg (S) measures how fast a particle sediments in a centrifuge, which depends on shape as well as mass, so S values do not add.
- Prokaryotes do have ribosomes (70S). Mitochondria and chloroplasts also have 70S ribosomes.
- "Blue-green algae" (cyanobacteria) are prokaryotes. Fungi are eukaryotes but not plants: chitin walls, no chlorophyll.
- A nucleoid is not a nucleus: it has no membrane.
- *Mycoplasma* has no cell wall but has sterols in its membrane, a favourite exception.

CHECK YOURSELF

– REMEMBER **Give the sedimentation coefficients of prokaryotic and eukaryotic ribosomes and their subunits.** 2 marks

Prokaryotic 70S = 50S + 30S. Eukaryotic (cytoplasmic) 80S = 60S + 40S.

– REMEMBER **Name three structures found only in eukaryotic cells.** 3 marks

Any three: nucleus with nuclear envelope, mitochondria, endoplasmic reticulum, Golgi apparatus, lysosomes, 9 + 2 flagella.

– UNDERSTAND **Why does penicillin kill bacteria but not human cells?** 2 marks

It blocks the synthesis of peptidoglycan, the bacterial wall polymer. Human cells have no peptidoglycan, so the drug has no target in them.

– APPLY **Find the A/V ratio of a spherical coccus 2 µm in diameter and compare it with a 1 µm coccus.** 3 marks

r = 1 µm, A/V = 3/r = 3 μm^{-1}. The 1 µm coccus has 6 μm^{-1}, twice as much surface per unit volume.

– ANALYZE **A cell has no nucleus, 70S ribosomes, no peptidoglycan, ether-linked membrane lipids and DNA on histone-like proteins. Which domain is it in, and which features rule out the others?** 4 marks

Archaea. No nucleus and 70S ribosomes rule out Eukarya; ether-linked lipids and no peptidoglycan rule out Bacteria.

– UNDERSTAND **Give two pieces of evidence for the endosymbiont theory.** 2 marks

Mitochondria and chloroplasts have their own circular DNA and 70S ribosomes, and they divide by fission like bacteria.

BEYOND THE SYLLABUS

The line between the two plans is less sharp than textbooks show. *Thiomargarita magnifica*, a bacterium described in 2022 from Caribbean mangroves, is about a centimetre long and keeps its DNA inside membrane-bound sacs. Asgard archaea from deep-sea sediments carry genes once thought to be found only in eukaryotes, and are studied as the closest living relatives of the first eukaryotic cell. Search "Asgard archaea" or "bacterial cytoskeleton FtsZ MreB". This is not for the exam.

1.3 Microbial diversity and types of microorganisms

Microbes are the most numerous and varied living things on Earth. One estimate puts the number of prokaryotic cells on the planet at 4–6 × 10^{30} (Whitman and colleagues, 1998), living everywhere from the soda water of Lonar Lake in Maharashtra to the human gut. This chapter is the map; each group gets its own chapter in Units 2 and 3.

The types of microorganisms

GROUP	CELL TYPE	KEY FEATURES	EXAMPLE
Bacteria	Prokaryotic	Peptidoglycan wall; cocci, rods, spirals	*Lactobacillus* in curd
Archaea	Prokaryotic	No peptidoglycan; ether-linked lipids; many in extreme or oxygen-free places	Methane makers in biogas plants
Fungi	Eukaryotic	Chitin wall; absorb food; yeasts are single cells, moulds grow as threads (hyphae)	*Saccharomyces*, *Penicillium*
Algae	Eukaryotic	Chlorophyll; usually cellulose wall	*Chlorella*, diatoms
Protozoa	Eukaryotic	No wall; usually motile; ingest food	*Plasmodium*, *Entamoeba*
Viruses	Not cells	DNA or RNA in a protein coat; multiply only inside host cells	SARS-CoV-2, rabies virus
Viroids, prions	Not cells	Viroid: naked circular RNA; prion: misfolded protein	Potato spindle tuber viroid

Helminthes") because their eggs and larvae are identified under the microscope; see Unit 2, *Protozoa and helminths*.

Ways in which microbes differ

Microbes differ in size (Figure 1.3.1), in shape (Unit 2), in where they get energy and carbon (light or chemicals, CO_2 or organic matter; Unit 4), in their need for oxygen, and in habitat, up to the extremophiles of hot springs and salt pans (Unit 2). Most of this diversity has never been grown in a lab: plate counts of soil or sea water recover only a small fraction (often quoted as about 1%) of the cells seen under the microscope. This gap is called the great plate count anomaly, and DNA-based methods (Chapter 1.5) were developed to see past it.

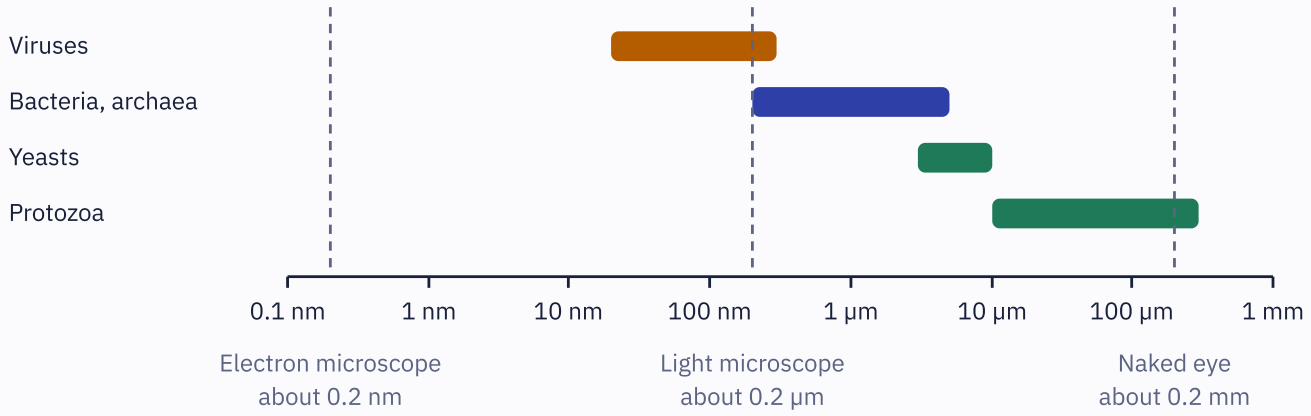


Figure 1.3.1 Typical size ranges on a logarithmic scale (each tick is ten times the last), with the smallest detail each way of seeing can resolve (dashed lines). Most viruses fall below the light-microscope limit and need the electron microscope; bacteria sit just above it; the largest protozoa reach the limit of the naked eye.

WORKED EXAMPLE

Question (6 marks). Name the group of each organism and say whether it is prokaryotic, eukaryotic or acellular: (a) *Spirulina*, sold as "blue-green alga" tablets; (b) *Saccharomyces cerevisiae*; (c) *Plasmodium falciparum*; (d) *Methanobrevibacter smithii* from the human gut; (e) tobacco mosaic virus; (f) *Chlorella*.

1. First ask: is it a cell? Only (e) is not; a virus is acellular.
2. Next ask: does the cell have a nucleus? (a) *Spirulina* does not: it is a cyanobacterium, a prokaryote, whatever the label says. (d) is also a prokaryote; it makes methane and lacks peptidoglycan, so it is an archaeon.
3. Then sort the eukaryotes by wall and nutrition: (b) wall of glucan and chitin, absorbs food: a fungus (yeast). (c) no wall, parasite of red blood cells: a protozoan. (f) chlorophyll and a cellulose wall: a green alga.

Answer: (a) bacterium, prokaryotic; (b) fungus, eukaryotic; (c) protozoan, eukaryotic; (d) archaeon, prokaryotic; (e) virus, acellular; (f) alga, eukaryotic.

EXAM TRAPS

- "Blue-green algae" are cyanobacteria: prokaryotes, not algae.
- Viruses are not prokaryotes. They are not cells at all: no ribosomes and no metabolism of their own.
- Yeasts and moulds are both fungi; some fungi switch between the two forms.

CHECK YOURSELF

– REMEMBER **List the major groups of microorganisms with one example each.** 4 marks

Bacteria (*E. coli*), archaea (methanogens), fungi (yeast), algae (*Chlorella*), protozoa (*Plasmodium*), viruses (SARS-CoV-2).

– UNDERSTAND **What is the great plate count anomaly?** 2 marks

Far fewer colonies grow on plates than cells are seen under the microscope, so most environmental microbes have never been cultured.

– APPLY **Using Figure 1.3.1, which can be resolved with a light microscope: SARS-CoV-2 (about 100 nm), *Mycoplasma* (about 0.3 µm), a yeast cell (5 µm)?** 3 marks

The yeast clearly. *Mycoplasma* only just, as a dot without detail. SARS-CoV-2 is below the 0.2 µm limit and needs an electron microscope.

– ANALYZE **A microbe has a nucleus and a wall, no chlorophyll, and grows as branched threads. Name its group and the feature that rules out each alternative.** 4 marks

A mould (fungus). No chlorophyll rules out algae; the nucleus rules out actinomycetes, which are thread-forming bacteria; the wall rules out protozoa.

– UNDERSTAND **Why are helminths taught in microbiology although they are not microbes?** 2 marks

They cause infections that are diagnosed by finding their microscopic eggs or larvae in samples.

BEYOND THE SYLLABUS

Projects such as the Earth Microbiome Project map diversity by sequencing DNA straight from soil, water and gut samples. One surprise is a large group of very small bacteria, the candidate phyla radiation, known almost only from their genomes. Giant viruses such as Mimivirus, described in 2003, are larger than some bacteria. None of this is for the exam.

1.4 Classification, taxonomy and identification of microbes

Taxonomy is the science of sorting living things into groups. It has three parts: classification (arranging organisms into groups by their similarities), nomenclature (giving each group its correct name) and identification (deciding which named group an unknown organism belongs to). Microbes are hard to classify because many look alike under the microscope and most reproduce without sex, so microbial taxonomy has moved from shape and staining to biochemistry, and now to DNA sequences.

You need: the prokaryote–eukaryote difference from Chapter 1.2.

Ranks and names

Organisms are placed in ranks, each inside the next: domain, phylum, class, order, family, genus, species (left of Figure 1.4.1). Below species, microbiologists use the **strain**, a pure culture descended from one isolate (*E. coli* K-12), the **serovar** or serotype, strains that differ in surface antigens (*E. coli* O157:H7), and the **biovar**, strains that differ in biochemistry.

Names follow the binomial system of Linnaeus: the genus with a capital letter, then the species epithet in lower case, both in italics (underlined when handwritten), as in *Escherichia coli*. After the first use, the genus may be shortened to *E. coli*. Names of bacteria and archaea follow the International Code of Nomenclature of Prokaryotes. A new species name becomes valid only when it is published in, or listed by, the *International Journal of Systematic and Evolutionary Microbiology*, and its type strain (the reference culture that carries the name) is deposited in public culture collections; India's include MTCC in Chandigarh and NCIM in Pune.

Names change as knowledge improves. In 2021 the phylum names were brought under the code, so Proteobacteria became Pseudomonadota and Firmicutes became Bacillota. Many books and question papers still use the old names; use the one your syllabus uses and mention the other.

From two kingdoms to three domains

Linnaeus recognised two kingdoms, plants and animals, and microbes were forced into one or the other. Ernst Haeckel (1866) added a third, Protista, for microorganisms. Robert Whittaker's five-kingdom system (1969) used cell type, cell number and mode of nutrition to give Monera (all prokaryotes), Protista, Fungi, Plantae and Animalia.

Carl Woese compared the sequences of small-subunit ribosomal RNA (16S rRNA in prokaryotes, 18S in eukaryotes) across many organisms. In 1977 he found that the prokaryotes formed two groups as different from each other as either is from eukaryotes, and in 1990 he and his colleagues proposed three domains, Bacteria, Archaea and Eukarya, above the level of kingdom. rRNA was a good choice because every cell has it, it does the same job everywhere, and it contains both slowly changing (conserved) and faster changing (variable) stretches, so it can compare distant and close relatives alike.

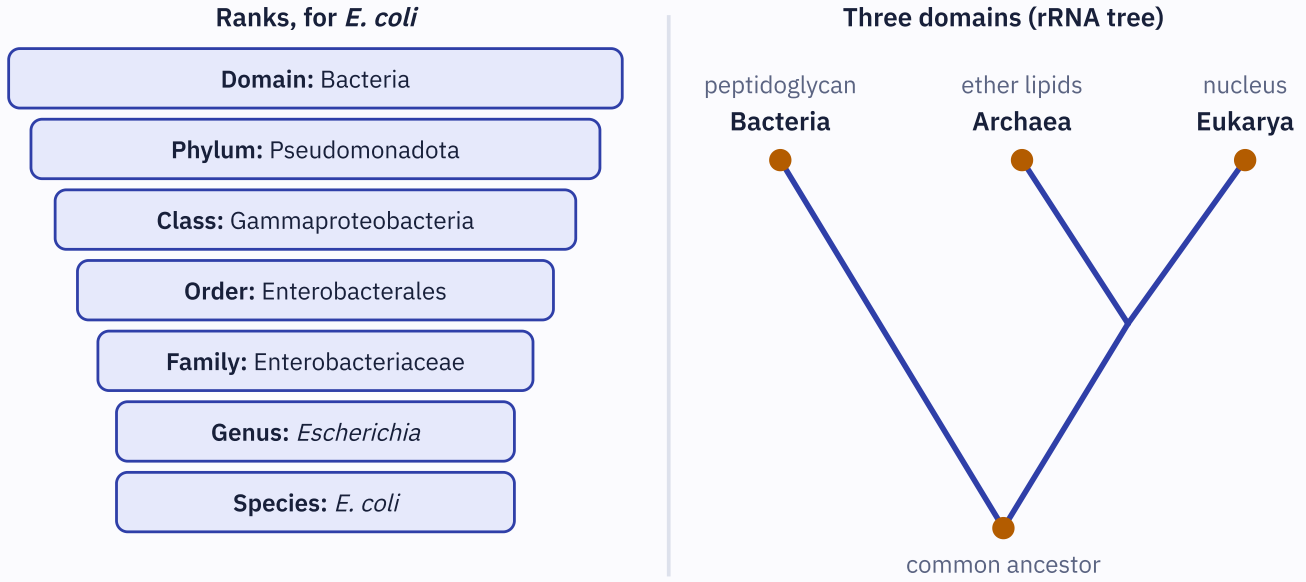


Figure 1.4.1 Left: each rank sits inside the one above it. Right: Woese's tree from rRNA sequences, with one hallmark above each domain name. Notice that Archaea and Eukarya share a branch point: archaea are more closely related to eukaryotes than to bacteria, even though archaea and bacteria are both prokaryotes.

Three ways to classify

Phenetic classification groups organisms by overall similarity in many observable characters (shape, staining, growth, biochemistry), all weighted equally. Its quantitative form is numerical taxonomy, developed by Sneath and Sokal: score 50 or more characters as + or −, calculate a similarity coefficient for every pair of strains, and join the most similar strains first into a tree-like diagram called a dendrogram. For two strains, let a = characters positive in both, b and c =

characters positive in only one, and d = characters negative in both. The simple matching coefficient S_SM and the Jaccard coefficient S_J are:

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S_SM = (a + d) / (a + b + c + d)
S_J  = a / (a + b + c)
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S_J leaves out shared negatives, because two strains can fail the same test for unrelated reasons. **Phylogenetic classification** groups organisms by evolutionary descent, read from gene sequences (16S rRNA, and now whole genomes). **Polyphasic taxonomy**, today's standard for describing a new species, combines phenotypic, chemical and genetic data (Chapter 1.5).

Identification

Identification uses only the few characters that separate known groups. A dichotomous key asks a series of two-way questions: Gram-positive or Gram-negative? Cocci or rods? Catalase-positive or negative? Each answer leads to the next question until one name remains. Gram-positive cocci in clusters that are catalase-positive are staphylococci; if they are also coagulase-positive, the organism is *Staphylococcus aureus*. The standard reference is Bergey's Manual. *Bergey's Manual of Determinative Bacteriology* is the identification guide; *Bergey's Manual of Systematic Bacteriology* describes classification and relationships, and continues online as *Bergey's Manual of Systematics of Archaea and Bacteria*.

WORKED EXAMPLE

Question (8 marks). Three isolates from curd were scored on ten tests. Calculate S_SM and S_J for each pair, and say which two isolates join first in a dendrogram.

TEST	1	2	3	4	5	6	7	8	9	10
A	+	+	−	+	−	+	+	−	−	+
B	+	+	−	+	−	+	−	−	−	+
C	−	+	+	−	+	−	+	+	−	−

1. **A and B.** Both positive in tests 1, 2, 4, 6, 10: a = 5. A only in test 7: b = 1, c = 0. Both negative in 3, 5, 8, 9: d = 4. S_SM = (5 + 4)/10 = 0.90; S_J = 5/(5 + 1 + 0) = 0.83.
2. **A and C.** a = 2 (tests 2, 7); b = 4 (1, 4, 6, 10); c = 3 (3, 5, 8); d = 1 (9). S_SM = 3/10 = 0.30; S_J = 2/9 = 0.22.
3. **B and C.** a = 1 (test 2); b = 4 (1, 4, 6, 10); c = 4 (3, 5, 7, 8); d = 1 (9). S_SM = 2/10 = 0.20; S_J = 1/9 = 0.11.
4. **Cluster.** A and B are most similar by either coefficient, so they join first, at 0.90 (S_SM). C then joins the A–B group at the average of its two values: (0.30 + 0.20)/2 = 0.25.

Conclusion: A and B are probably strains of one species; C is a different organism. A real study would score many more tests and confirm the grouping with 16S rRNA sequencing.

EXAM TRAPS

- Bergey's *Determinative* manual is for identification; the *Systematic* manual is for classification. Examiners ask the difference.
- Write *Escherichia coli* or *E. coli*: capital genus, small species, italic or underlined. "E. Coli" loses marks.
- Classification builds the groups; identification places an unknown into a group that already exists.
- State which coefficient you used: S_SM counts shared negatives (d), S_J does not, so their values differ.
- Domains are above kingdoms, not another name for them.

CHECK YOURSELF

– REMEMBER **List the taxonomic ranks from domain to species.** 2 marks

Domain, phylum, class, order, family, genus, species.

– REMEMBER **Name Whittaker's five kingdoms.** 2 marks

Monera, Protista, Fungi, Plantae, Animalia.

– UNDERSTAND **Why is 16S rRNA suited to building a tree of all cellular life?** 3 marks

All cells have it; it has the same function everywhere; its conserved regions allow alignment across distant groups while its variable regions separate close relatives.

– APPLY **Two strains are both positive in 6 tests, both negative in 2, and differ in 2 (each positive in one). Find S_SM and S_J.** 3 marks

a = 6, b = 1, c = 1, d = 2. S_SM = (6 + 2)/10 = 0.80; S_J = 6/(6 + 1 + 1) = 0.75.

– ANALYZE **An isolate is a Gram-positive coccus in clusters, catalase-positive and coagulase-negative. What can you conclude, and why is the key not finished?** 4 marks

It is a coagulase-negative staphylococcus, not *S. aureus*. Several species fit (for example *S. epidermidis*, *S. saprophyticus*), so further tests such as novobiocin sensitivity are needed.

– UNDERSTAND **How does a strain differ from a species?** 2 marks

A species is a group of similar strains; a strain is one pure-culture line within a species, descended from a single isolate.

BEYOND THE SYLLABUS

Since 2018 the Genome Taxonomy Database (GTDB) has reclassified bacteria and archaea from whole-genome trees, and in 2022 the SeqCode was launched so that microbes known only from DNA sequences can receive formal names. Uncultured organisms are given provisional "*Candidatus*" names. Search "GTDB taxonomy" or "SeqCode prokaryotes" to read further. This is not for the exam.

1.5 Biochemical and molecular characterization of microbes

Two bacteria can look identical after a Gram stain; their enzymes and genes tell them apart. Biochemical characterization tests what an organism does: which sugars it ferments, which enzymes it makes, which products it forms. Molecular characterization reads what it is: the base composition of its DNA, the sequence of marker genes, or its whole genome. Together with shape and staining they form the polyphasic approach of Chapter 1.4.

Biochemical tests

Most tests detect one enzyme or pathway, usually by a colour change. They use a pure culture 18–24 hours old, because mixed or old cultures give confusing results.

TEST	WHAT IT DETECTS	POSITIVE RESULT	EXAMPLE
Catalase	Enzyme splitting H_2O_2 into water and oxygen	Bubbles with 3% H_2O_2	*Staphylococcus* +, *Streptococcus* –
Oxidase	Cytochrome c oxidase	Purple colour within seconds	*Pseudomonas* +, *E. coli* –
Sugar fermentation	Acid, and gas, from a sugar	Phenol red turns yellow; gas in a Durham tube	*E. coli*: lactose, acid and gas
Indole	Tryptophan split to indole	Red ring with Kovács' reagent	*E. coli* +
Methyl red (MR)	Mixed-acid fermentation, strong acid	Red	*E. coli* +
Voges–Proskauer (VP)	Acetoin, from butanediol fermentation	Pink-red with α-naphthol and KOH	*Klebsiella pneumoniae* +
Citrate	Citrate used as the only carbon source	Simmons citrate agar turns blue	*Klebsiella* +, *E. coli* –
Urease	Urea split to ammonia and CO_2	Pink (alkaline)	*Proteus* +
H_2S	Sulfur compounds reduced to H_2S	Black precipitate in TSI agar	Most *Salmonella* +
Coagulase	Enzyme that clots plasma	Clot	*S. aureus* +

Indole, MR, VP and citrate together are the IMViC tests, used to separate coliform bacteria: *E. coli* gives + + – – and *Klebsiella pneumoniae* – – + +.

Kits, machines and chemical fingerprints

Miniaturised kits run many tests at once. The API 20E strip for enteric bacteria has 20 small cups of dried substrates. With the oxidase test, the 21 results are split into seven groups of three; a positive scores 1, 2 or 4 by its place in the group, and the seven group sums form a profile number that is looked up in the maker's database (worked example 1). Automated systems such as VITEK 2 read colour changes in sealed cards by machine.

Chemotaxonomy uses chemical make-up instead of reactions. Fatty acid methyl ester (FAME) analysis profiles membrane fatty acids by gas chromatography. MALDI-TOF mass spectrometry identifies a colony in minutes from the mass pattern of its abundant proteins, mostly ribosomal proteins of about 2–20 kDa, matched against a reference library; many hospital labs now use it in place of long biochemical panels.

Molecular characterization

G + C content. The mol% G + C of DNA is (G + C) ÷ (A + T + G + C) × 100. Strains of one species differ little, so a large difference rules out a close relationship, though similar values do not prove one. It is measured from the melting temperature T_m, at which half the DNA strands have separated: G·C pairs have three hydrogen bonds and A·T pairs two, so G + C-rich DNA melts higher. In standard saline citrate, Marmur and Doty's relation is $T_m = 69.3 + 0.41 \times$ (mol% G + C), with T_m in °C.

DNA–DNA hybridization. DNA from two strains is melted, mixed and allowed to re-pair. At least 70% re-pairing was the classic test for the same species.

16S rRNA gene sequencing. The gene is about 1,500 base pairs long, with nine variable regions (V1–V9) between conserved ones (Figure 1.5.1). Universal primers bind the conserved ends, PCR copies the gene, and the sequence is compared with databases such as NCBI BLAST or EzBioCloud. As a rule of thumb, 98.7% identity or more suggests the same species, and below about 95% a different genus. The gene is too conserved to separate some close species: *E. coli* and *Shigella* are nearly identical in 16S.

Genomes and typing. Average nucleotide identity (ANI) between two whole genomes, 95–96% or more for the same species, has largely replaced DNA–DNA hybridization. To tell strains apart in an outbreak, labs use multilocus sequence typing (MLST, sequences of seven housekeeping genes), pulsed-field gel electrophoresis and, increasingly, whole-genome sequencing.

The as-yet-unculturables. Culture-independent methods work on DNA or RNA taken straight from a sample: 16S amplicon sequencing lists which groups are present; shotgun metagenomics sequences all the DNA and rebuilds genomes from it; fluorescence in situ hybridization (FISH)

lights up chosen groups with fluorescent rRNA probes (Chapter 1.7); and quantitative PCR counts copies of a target gene.

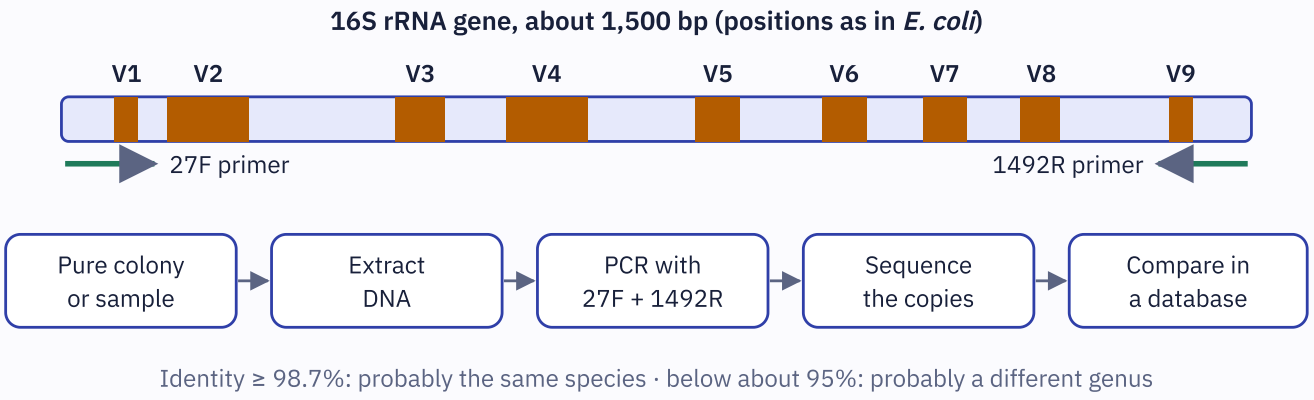


Figure 1.5.1 Top: the variable regions V1–V9 (orange) lie between conserved stretches (blue), where the universal primers bind. Bottom: the steps from colony to name. Notice that the primers sit in conserved DNA, so the same pair copies the gene from almost any bacterium, while the variable regions carry the information that tells species apart.

WORKED EXAMPLE 1: AN API 20E PROFILE

Question (5 marks). An oxidase-negative, lactose-fermenting rod gives these API 20E results, in strip order: ONPG +, ADH –, LDC +, ODC +, CIT –, H_2S –, URE –, TDA –, IND +, VP –, GEL –, GLU +, MAN +, INO –, SOR +, RHA +, SAC –, MEL +, AMY –, ARA +, OX –. Find the profile number.

1. Split into groups of three; score 1, 2, 4 for a positive in the first, second, third place.
2. ONPG, ADH, LDC = 1 + 0 + 4 = 5. ODC, CIT, H_2S = 1 + 0 + 0 = 1. URE, TDA, IND = 0 + 0 + 4 = 4. VP, GEL, GLU = 0 + 0 + 4 = 4.
3. MAN, INO, SOR = 1 + 0 + 4 = 5. RHA, SAC, MEL = 1 + 0 + 4 = 5. AMY, ARA, OX = 0 + 2 + 0 = 2.
4. Profile number: 5 144 552. The database lookup gives *E. coli*, as the key results already suggest: ONPG and indole positive; citrate, H_2S, urease and VP negative.

WORKED EXAMPLE 2: 16S IDENTITY AND G + C

Question (5 marks). An isolate's 16S rRNA gene, 1,460 bp after alignment, differs from its closest type strain at 22 positions. Its DNA has T_m = 91.0 °C in standard saline citrate. Find (a) the percentage identity, (b) whether it is likely to be the same species, (c) its mol% G + C.

1. (a) Identity = (1,460 − 22) ÷ 1,460 × 100 = 1,438 ÷ 1,460 × 100 = 98.49%.
2. (b) 98.49% is below 98.7%, so it is probably a different species of the same genus (it is well above 95%). Confirm with ANI from genome sequences.
3. (c) mol% G + C = (T_m − 69.3) ÷ 0.41 = (91.0 − 69.3) ÷ 0.41 = 21.7 ÷ 0.41 = 52.9%.

EXAM TRAPS

- Catalase on colonies from blood agar can give a false positive, because red blood cells contain catalase.
- Do the oxidase test with a platinum loop or wooden stick; iron in a nichrome loop can give a false positive.
- MR and VP are usually opposite for one organism: mixed-acid and butanediol fermentation are alternative routes.
- Low 16S identity is strong evidence of a different species; high identity is weak evidence of the same one.
- Similar G + C content does not prove a relationship; very different values rule one out.

CHECK YOURSELF

– REMEMBER **What do the letters IMViC stand for, and what is the IMViC pattern of *E. coli*?** 3 marks

Indole, Methyl red, Voges–Proskauer, Citrate (the "i" is for easy saying). *E. coli* is + + − −.

– UNDERSTAND **Why does G + C-rich DNA melt at a higher temperature?** 2 marks

A G·C pair is held by three hydrogen bonds and an A·T pair by two, and G·C pairs also stack more strongly, so more heat is needed to separate the strands.

– APPLY **DNA from an isolate melts at 85.0 °C in standard saline citrate. Find its mol% G + C.** 2 marks

(85.0 − 69.3) ÷ 0.41 = 15.7 ÷ 0.41 = 38.3%.

– APPLY **In an API 20E strip, ODC –, CIT +, H_2S +. What digit does this group give?** 1 mark

0 + 2 + 4 = 6.

– ANALYZE **Two isolates share 99.6% 16S identity but their genomes have an ANI of 91%. Are they the same species? Explain.** 3 marks

No. ANI below 95–96% shows different species. The 16S gene changes too slowly to separate close species, so its high identity does not decide the question.

– UNDERSTAND **How can we study microbes that cannot be cultured?** 3 marks

Extract DNA or RNA directly from the sample and use 16S amplicon sequencing, shotgun metagenomics, FISH with rRNA probes or quantitative PCR.

BEYOND THE SYLLABUS

Whole-genome sequencing is now routine in public-health labs; during COVID-19, India's INSACOG network tracked SARS-CoV-2 variants this way. Portable nanopore sequencers read DNA in the field, and resistance-gene databases let labs predict drug resistance from a genome (Unit 5, *Antimicrobial resistance*). Search "MALDI-TOF identification" or "shotgun metagenomics" to read more. This is not for the exam.

1.6 Light microscopy: bright field, dark field, phase contrast

A light microscope uses visible light and glass lenses to form an enlarged image. Two numbers describe it: magnification, how many times bigger the image is, and resolution, the smallest distance between two points that can still be seen as two. Magnification without resolution only gives a bigger blur. Bright-field microscopy is the everyday method; dark-field and phase-contrast microscopy change how the specimen is lit, so that living, unstained cells become visible.

The compound microscope

Light goes from the lamp through the **condenser**, which focuses it on the specimen and has an iris diaphragm to control the cone of light. It then passes through the **objective**, which forms a magnified image, and the **eyepiece** (ocular), which magnifies that image again. Student microscopes usually have 4×, 10×, 40× and 100× (oil-immersion) objectives with a 10×

eyepiece. Total magnification is the objective's magnification times the eyepiece's: 100 × 10 = 1000× with the oil objective.

The objectives are parfocal: when one is in focus, the next is nearly in focus too. The working distance between objective and slide shrinks as magnification rises, to a fraction of a millimetre for the 100× objective.

Resolution and numerical aperture

Light is a wave and spreads a little as it passes small objects (diffraction), so each point of the specimen becomes a small blurred disc in the image. Two points are resolved only if their discs do not overlap too much. Ernst Abbe's limit gives the smallest resolvable distance d:

```
d = 0.5 λ / NA          NA = n sin θ
```

Here λ is the wavelength of light, NA the numerical aperture of the objective, n the refractive index of the medium between specimen and objective (air 1.00, immersion oil about 1.515) and θ half the angle of the cone of light that enters the objective. Some books use d = 0.61 λ / NA (the Rayleigh criterion); the numbers differ slightly, the idea is the same. A smaller d means better resolution, so resolution improves with shorter wavelength and larger NA.

Because sin θ cannot exceed 1, a dry objective (n = 1) always has NA below 1, about 0.95 at best. Immersion oil raises NA to about 1.25–1.4: its refractive index matches glass, so rays that leave the slide at wide angles are not bent away by an air gap but enter the objective. With green light (λ = 550 nm) and NA 1.25, d ≈ 0.22 µm, so a light microscope cannot resolve detail finer than about 0.2 µm. Useful magnification is about 500–1000 × NA; beyond that the image grows but shows no new detail ("empty magnification").

Bright-field microscopy

The full cone of light from the condenser enters the objective, so the specimen looks darker than a bright background. Most cells are almost transparent, with a refractive index close to water, so they show little contrast unless they are stained (Chapter 1.8), and staining kills them. Closing the iris diaphragm adds a little contrast but costs resolution.

Dark-field microscopy

An opaque disc, the **patch stop**, sits below the condenser so that only a hollow cone of light reaches the specimen. The cone is too wide to enter the objective (the condenser's NA must exceed the objective's), so an empty field looks black. Objects in the specimen scatter some light into the objective and shine against the dark background, like dust in a sunbeam. Dark-field shows live, unstained cells and their movement, and it reveals cells thinner than the resolution

limit because they still scatter light. It is the classic method for the thin syphilis spirochaete *Treponema pallidum*. It shows outlines, not internal detail.

Phase-contrast microscopy

A living cell has a slightly higher refractive index than the water around it, so light passing through it is slowed and comes out slightly behind in phase. Eyes and cameras sense brightness, not phase. Frits Zernike's phase-contrast microscope (Nobel Prize in Physics, 1953) turns phase differences into brightness differences:

1. An annular diaphragm (a ring-shaped opening) below the condenser gives a hollow cone of light.
2. Light that passes the specimen undeviated (the surround light) forms a bright ring behind the objective, where a phase plate carries a matching phase ring.
3. Light diffracted by the specimen spreads out and mostly misses the ring. For a thin, transparent object it is about a quarter wavelength (λ/4) behind the surround light.
4. The phase ring shifts the surround light by a further λ/4 and dims it, so the two are now about half a wavelength (λ/2) apart.
5. Where they meet in the image they interfere destructively: the cell looks dark on a grey background.

Phase contrast shows living cells dividing, bright "refractile" endospores, inclusion bodies and moving protozoa without stain. Bright halos around cells are a normal artefact. Differential interference contrast (DIC, or Nomarski) is a related method that gives a shadowed, almost three-dimensional look.

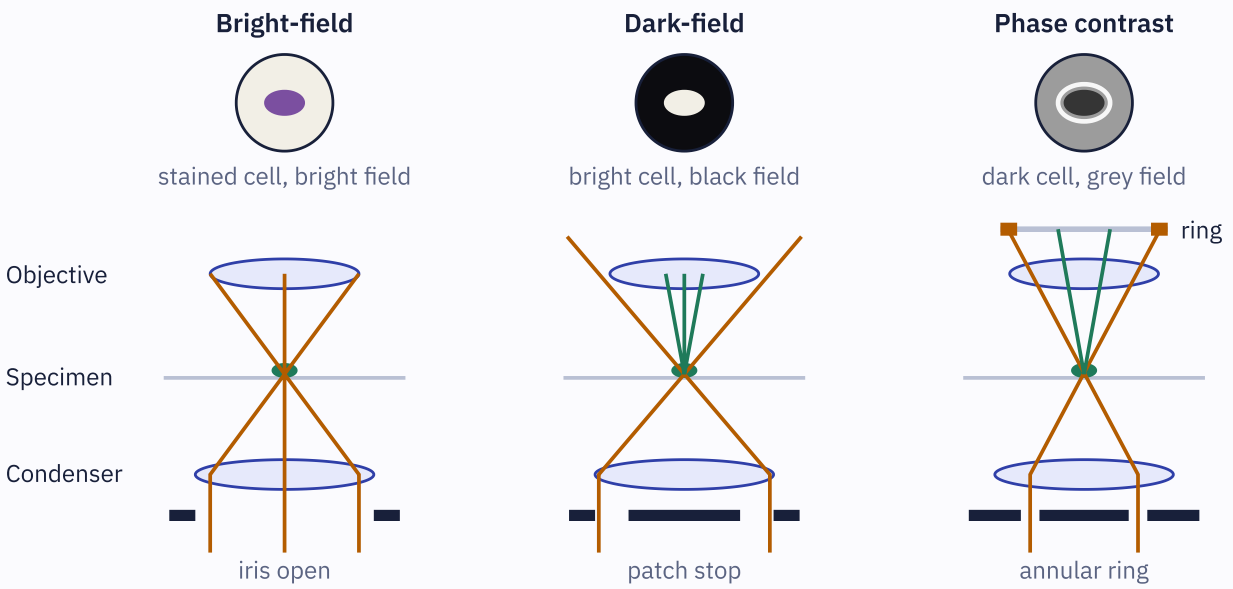


Figure 1.6.1 Light (orange) comes up from the lamp. Bright-field: the whole cone enters the objective. Dark-field: the patch stop makes a hollow cone that misses the objective, so only light scattered by the specimen (green) is collected. Phase contrast: the hollow cone from the annular ring lands on the phase ring, while the diffracted light (green) passes beside it; the two then interfere to give contrast.

WORKED EXAMPLE

Question (6 marks). Using d = 0.5 λ / NA, find the resolution of (a) a 40× dry objective, NA 0.65, with green light of 550 nm; (b) a 100× oil objective, NA 1.25, same light; (c) the oil objective with blue light of 450 nm. (d) What is the useful magnification range for (b)? (e) Can (a) show the width of a rod 0.5 µm wide?

1. (a) d = 0.5 × 550 ÷ 0.65 = 275 ÷ 0.65 = 423 nm ≈ 0.42 µm.
2. (b) d = 275 ÷ 1.25 = 220 nm = 0.22 µm. Oil roughly halves the resolvable distance.
3. (c) d = 0.5 × 450 ÷ 1.25 = 225 ÷ 1.25 = 180 nm = 0.18 µm. Shorter wavelength, finer detail.
4. (d) 500 × 1.25 to 1000 × 1.25 = 625× to 1250×. So 100 × 10 = 1000× is useful; a 20× eyepiece giving 2000× would be empty magnification.
5. (e) Yes, just: 0.5 µm is larger than d = 0.42 µm. Flagella, about 20 nm thick, are far below d for any light objective.

EXAM TRAPS

- Magnification is not resolution. A stronger eyepiece without a higher NA adds no detail.
- A dry objective cannot reach NA 1, because n = 1 for air and sin θ is less than 1.
- The 100× objective needs oil; without it the image is dim and blurred. Do not put oil on the 40× dry objective.
- Dark-field needs a condenser NA higher than the objective NA.
- Phase contrast uses no stain, and the bright halo is an artefact, not a capsule.

CHECK YOURSELF

– REMEMBER **Write the formula for resolving power and define numerical aperture.** 2 marks

d = 0.5 λ / NA (or 0.61 λ / NA); NA = n sin θ, where n is the refractive index of the medium and θ half the angle of the light cone entering the objective.

– REMEMBER **Which microscope is used to look for *Treponema pallidum* in a fresh sample?** 1 mark

The dark-field microscope.

– UNDERSTAND **How does immersion oil improve resolution?** 3 marks

Oil has about the same refractive index as glass (1.515), so wide-angle rays are not bent away at an air gap. More of the light cone enters the objective, raising NA = n sin θ and lowering d.

– APPLY **At 40×, 40 ocular divisions line up with 10 stage-micrometer divisions of 10 µm each. A yeast cell spans 2 ocular divisions. How big is it?** 3 marks

10 × 10 µm = 100 µm = 40 ocular divisions, so 1 division = 2.5 µm. The cell is 2 × 2.5 = 5 µm.

– APPLY **A cell 2 µm thick has refractive index 1.37 in water (1.33). Find the optical path difference and express it as a fraction of 550 nm.** 3 marks

OPD = (1.37 − 1.33) × 2,000 nm = 80 nm; 80 ÷ 550 ≈ 0.15, about one-seventh of a wavelength. Too small to see as brightness, which is why phase contrast is needed.

– ANALYZE **You need to watch the internal structures of live, unstained protozoa, and separately to check pus for thin motile spirochaetes. Which microscope for each, and why?** 4 marks

Phase contrast for the protozoa, because it turns refractive-index differences inside live cells into contrast. Dark-field for spirochaetes, because they are too thin and faint for bright-field but scatter light and show their motion against the black field.

BEYOND THE SYLLABUS

Super-resolution methods such as STED and PALM/STORM get below the 0.2 µm limit using fluorescent molecules (Nobel Prize in Chemistry, 2014; see Chapter 1.7). At the other end, low-cost paper microscopes such as the Foldscope and smartphone microscopes bring bright-field microscopy to schools and field clinics. Search "Abbe diffraction limit" or "Foldscope" to read more. This is not for the exam.

1.7 Fluorescence and electron microscopy

Bright-field microscopy has two weaknesses: poor contrast and a resolution of about 0.2 µm. Fluorescence microscopy fixes the first: chosen molecules or cells glow in colour against a black background, so single bacteria stand out and specific targets can be labelled. Electron microscopy fixes the second: an electron beam has a wavelength about 100,000 times shorter than visible light, so it can show viruses, ribosomes and membranes.

You need: resolution and numerical aperture from Chapter 1.6.

Fluorescence and the epifluorescence microscope

A fluorochrome (fluorescent dye) absorbs light of one wavelength and, within nanoseconds, gives off light of a longer wavelength. Some energy is lost in between, so the emitted light is always redder than the exciting light; the gap between the two peak wavelengths is the Stokes shift. Photon energy is $E = hc/\lambda$, where h is Planck's constant and c the speed of light; in handy units, $E\ (\text{eV}) \approx 1240 \div \lambda\ (\text{nm})$.

Modern instruments light the specimen from above, through the objective (epi-illumination), so the objective also acts as the condenser (Figure 1.7.1):

1. A mercury lamp or LED gives intense short-wavelength light.
2. An excitation filter passes only the wavelength that excites the dye, for example blue light for fluorescein (FITC).
3. A dichroic mirror reflects that light down through the objective onto the specimen.
4. The dye's longer-wavelength emission returns up through the objective, and the dichroic mirror lets it pass.
5. An emission (barrier) filter removes any stray excitation light, so only the glow reaches the eye or camera.

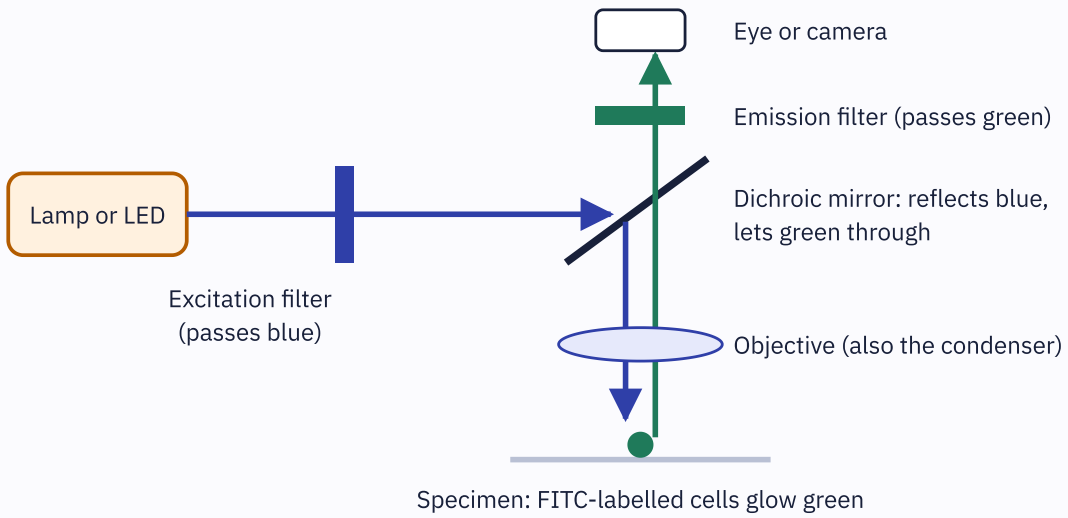


Figure 1.7.1 Blue excitation light (left) is reflected down onto the specimen; green emitted light travels up through the mirror and the emission filter. Notice that the two filters and the mirror together keep the bright exciting light away from the eye, which is why fluorescing cells appear on a black background.

What fluorescence is used for

- **Tuberculosis smears.** Auramine O stains the waxy wall of mycobacteria, which glow yellow-green and can be scanned at lower magnification, and so faster, than in Ziehl–Neelsen smears (Chapter 1.9). LED fluorescence microscopes are now common in TB laboratories.
- **Nucleic-acid stains.** DAPI (blue) and acridine orange bind DNA; DAPI is used to count all the bacteria in a water sample.
- **Immunofluorescence.** Antibodies tagged with a fluorochrome such as FITC find one microbe or antigen. In the direct method the labelled antibody binds the microbe; in the indirect method an unlabelled antibody binds first and a labelled anti-antibody binds to it, giving a stronger signal. The direct test is used to detect rabies virus in brain tissue.
- **Other labels.** Live/dead stain pairs, FISH probes for rRNA (Chapter 1.5), and green fluorescent protein (GFP) fused to bacterial proteins to follow them in living cells.

Confocal microscopy. An ordinary fluorescence image collects light from above and below the focal plane, so thick specimens look hazy. The confocal microscope scans a laser spot across the specimen and puts a pinhole in front of the detector to block out-of-focus light. Each scan is a sharp optical section, and a computer stacks sections taken at different depths into a 3-D image. It is used to study biofilms (Unit 6) and to locate structures inside cells.

Electron microscopy

Electrons are released from a heated tungsten filament or a field-emission tip and accelerated by a high voltage V. Their wavelength is $\lambda = h / \sqrt{(2meV)}$, where m and e are the electron's mass and charge; at 100 kV this is about 0.0037 nm. Electromagnetic coils act as lenses, and the column is kept under vacuum because air molecules would scatter the beam. So specimens must be dry and dead, except in cryo-electron microscopy, which images them frozen.

Transmission electron microscope (TEM). Electrons pass through a very thin specimen. Cells are fixed (glutaraldehyde, osmium tetroxide), dehydrated, embedded in resin and cut into sections 20–100 nm thick with an ultramicrotome. Heavy-metal stains (uranyl acetate, lead citrate) scatter electrons, so dense parts look dark on the screen or camera. Viruses are often negatively stained: the background is filled with electron-dense phosphotungstic acid or uranyl acetate, so particles appear light on dark. Shadowing (spraying metal at an angle) and freeze-fracture (splitting frozen cells along their membranes) show surfaces and membranes. The instrument resolves about 0.2 nm, though stained biological sections show detail only down to a few nanometres. The image is a 2-D view of internal structure.

Scanning electron microscope (SEM). A fine beam scans the surface of a whole specimen that has been fixed, dried and coated with a thin layer of gold or gold–palladium. The beam knocks secondary electrons out of the surface; a detector collects them and builds a 3-D-looking picture of the surface. Its resolution, a few nanometres, is poorer than TEM's, but it shows shape and arrangement, such as cocci in chains or a biofilm on a catheter. Both instruments give black-and-white images; coloured pictures are false-coloured afterwards.

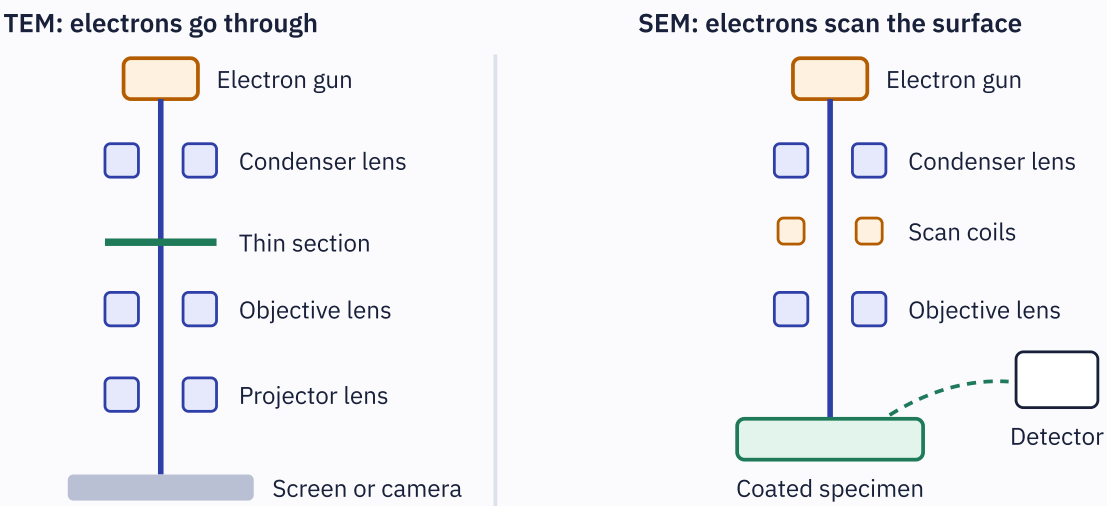


Figure 1.7.2 Both columns are under vacuum and use electromagnetic lenses (small squares). In the TEM the image is formed by electrons that pass through a thin section; in the SEM the scan coils (orange) sweep the beam over a coated specimen, and a detector collects the secondary electrons knocked out of its surface.

WORKED EXAMPLE 1: A FLUOROCHROME

Question (4 marks). DAPI absorbs best at about 358 nm and emits at about 461 nm. Find (a) the Stokes shift, (b) the energy of each photon in eV, (c) the energy lost per molecule, and (d) say which filters to use.

1. (a) 461 − 358 = 103 nm.
2. (b) Absorbed: 1240 ÷ 358 = 3.46 eV. Emitted: 1240 ÷ 461 = 2.69 eV.
3. (c) 3.46 − 2.69 = 0.77 eV, lost as heat before emission.
4. (d) An ultraviolet excitation filter (about 360 nm), a dichroic mirror that reflects ultraviolet and passes blue, and a blue emission filter (about 460 nm).

WORKED EXAMPLE 2: WHY ELECTRONS RESOLVE MORE

Question (6 marks). (a) Find the wavelength of electrons accelerated through 100 kV, using h = 6.63×10^{-34} J s, m = 9.11×10^{-31} kg, e = 1.60×10^{-19} C. (b) Compare it with green light (550 nm). (c) Estimate the resolution if lens defects limit the TEM to NA ≈ 0.01.

1. (a) $2meV = 2 \times 9.11 \times 10^{-31} \times 1.60 \times 10^{-19} \times 1.00 \times 10^{5} = 2.92 \times 10^{-44}$; its square root is 1.71×10^{-22}. $\lambda = 6.63 \times 10^{-34} \div 1.71 \times 10^{-22} = 3.88 \times 10^{-12}$ m = 0.0039 nm. (Correcting for relativity, since the electrons move at over half the speed of light, gives 0.0037 nm.)
2. (b) $550 \div 0.0037 \approx 1.5 \times 10^{5}$: the wavelength is about 150,000 times shorter.
3. (c) $d = 0.5 \lambda / NA = 0.5 \times 0.0037 \div 0.01 \approx 0.19$ nm, close to the 0.2 nm usually quoted. Electromagnetic lenses have large aberrations, so only a very narrow cone of electrons can be used; that, not the wavelength, sets the limit.

EXAM TRAPS

- The colour you see is the emission colour, which always has a longer wavelength than the excitation light.
- The dichroic mirror reflects the short excitation wavelength and transmits the longer emission.
- TEM: thin section, internal detail, 2-D. SEM: whole specimen, surface, 3-D look. Do not swap them.
- Conventional EM cannot show living cells (vacuum and fixation), and drying can create artefacts.
- Negative staining stains the background, not the virus.

CHECK YOURSELF

– REMEMBER **What is a fluorochrome? Name two used in microbiology.** 2 marks

A dye that absorbs light of one wavelength and emits light of a longer wavelength; for example auramine O, DAPI, acridine orange, FITC.

– REMEMBER **Why must the column of an electron microscope be under vacuum?** 2 marks

Air molecules would scatter and absorb the electron beam.

– UNDERSTAND **Why do fluorescing cells appear on a black background?** 2 marks

The dichroic mirror and emission filter block the excitation light, so only light emitted by the dye reaches the eye; unlabelled areas emit nothing.

– UNDERSTAND **How does indirect immunofluorescence differ from the direct method?** 2 marks

Direct: a labelled antibody binds the antigen. Indirect: an unlabelled antibody binds the antigen, then a labelled anti-antibody binds it, amplifying the signal.

– APPLY **Estimate the electron wavelength at 50 kV using $\lambda \approx 1.226 / \sqrt{V}$ nm.** 2 marks

√50,000 = 223.6; λ = 1.226 ÷ 223.6 = 0.0055 nm (0.0054 nm with the relativistic correction).

– ANALYZE **Choose a method to (a) see the shape of a virus particle, (b) count all bacteria in pond water, (c) see the 3-D arrangement of cells in a living biofilm, (d) see membranes inside a cyanobacterium. Justify each.** 4 marks

(a) TEM with negative staining: viruses are below light resolution. (b) Epifluorescence with DAPI: every cell glows and can be counted. (c) Confocal microscopy: optical sections of a thick, hydrated sample. (d) TEM of thin sections: internal membranes need nanometre resolution.

BEYOND THE SYLLABUS

Super-resolution fluorescence (STED, PALM, STORM) resolves tens of nanometres with visible light and won the 2014 Nobel Prize in Chemistry. Cryo-electron microscopy, which images molecules frozen in thin ice without stain, now reaches near-atomic detail (Nobel Prize in Chemistry, 2017), and cryo-electron tomography shows proteins inside whole bacterial cells. Search "cryo-electron tomography bacteria" to read more. This is not for the exam.

1.8 Staining: simple, Gram and differential stains

Most bacteria are colourless and almost transparent, so they are stained: a coloured dye binds to parts of the cell and gives contrast in the bright-field microscope. A simple stain uses one dye to show size, shape and arrangement. A differential stain uses two or more dyes in sequence and splits bacteria into groups that react differently. The most important is the Gram stain, devised by the Danish physician Hans Christian Gram in 1884. It is the first test done on almost any unknown bacterium, and often the first result a hospital laboratory reports.

You need: bright-field microscopy and oil immersion from Chapter 1.6.

Making a smear

1. Mix a little of a colony into a small drop of water on a clean, grease-free slide, or spread a loopful of broth culture. Keep it thin: a thick smear does not decolourize evenly.
2. Let it air-dry completely.
3. Heat-fix by passing the slide, smear side up, through a flame two or three times, or fix it with methanol. Fixing kills the cells, sticks them to the slide and lets dyes in. Too much heat distorts cells, and capsule and flagella stains (Chapter 1.9) use no heat at all.

How dyes stain

A dye is a coloured organic salt; its coloured part is the chromophore. In **basic (cationic) dyes** the chromophore carries a positive charge: methylene blue, crystal violet, safranin, malachite green, basic fuchsin. Bacterial surfaces and nucleic acids are negatively charged at near-neutral pH, so basic dyes bind to the cell. In **acidic (anionic) dyes** the chromophore is negative: eosin, nigrosin, Congo red. The cell repels them, so they colour the background instead. This is **negative staining**; it needs no heat-fixing, causes little shrinkage and shows true cell size. India ink is used the same way, though it is a suspension of carbon particles rather than a dye.

Multi-step stains add three kinds of reagent. A **mordant** increases the binding of a dye or forms an insoluble complex with it (Gram's iodine, tannic acid). A **decolourizer** removes dye from some cells but not others (alcohol, acetone, acid-alcohol). A **counterstain** of contrasting colour stains the cells that were decolourized.

Simple staining

Cover the fixed smear with one basic dye, such as methylene blue for about a minute or crystal violet for 20–60 seconds. Rinse gently with water, blot dry and examine under oil immersion. Every cell takes the same colour. The simple stain shows shape (cocci, rods, spirals) and arrangement (pairs, chains, clusters), which Unit 2 describes in *Bacterial morphology and cell structure*.

The Gram stain

1. **Primary stain:** crystal violet, 1 minute, then rinse. All cells are purple.
2. **Mordant:** Gram's iodine, 1 minute, then rinse. Crystal violet and iodine form a large crystal violet–iodine (CV–I) complex inside the cells. All cells are still purple.

3. **Decolourizer:** 95% ethanol, or acetone–alcohol, dropped on until the run-off is almost clear (about 10–20 seconds for ethanol, a few seconds for acetone–alcohol), then rinse at once. This is the critical step. Gram-positive cells stay purple; Gram-negative cells become colourless.
4. **Counterstain:** safranin, 30–60 seconds, rinse and blot. Gram-negative cells turn pink-red; Gram-positive cells stay purple, which hides the pink.

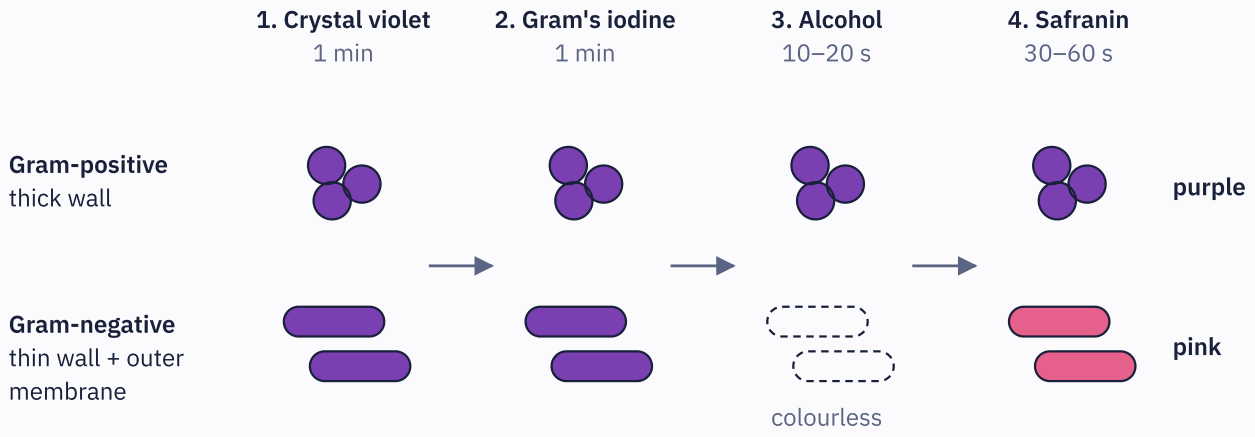


Figure 1.8.1 The colour of each cell type after each step. Notice that the two types look identical until step 3: the alcohol is the only step that tells them apart, and safranin only makes the decolourized Gram-negative cells visible again.

Why it works. The difference lies in the cell wall (Unit 2, *Bacterial cell wall: Gram-positive and Gram-negative*). A Gram-positive cell has a thick, many-layered peptidoglycan wall; alcohol dehydrates it and shrinks its pores, trapping the CV–I complex inside. A Gram-negative cell has a thin peptidoglycan layer under a lipid-rich outer membrane; alcohol dissolves the outer membrane, and the thin wall cannot hold the complex, which washes out.

Good practice. Use a young culture (18–24 hours), because older Gram-positive cells with damaged walls may stain pink (Gram-variable). Keep the smear thin, and stain a known Gram-positive (*Staphylococcus aureus*) and Gram-negative (*E. coli*) control on the same slide. Some organisms do not fit: *Mycoplasma* has no wall and does not give a true Gram reaction, and mycobacteria resist the stain because of their waxy wall (Chapter 1.9).

Differential stains

The Gram stain is the most used differential stain. Two more are taught with the special stains in the next chapter: the acid-fast stain, which picks out mycobacteria by their waxy wall, and the endospore stain, which picks out spores. All three follow one pattern: a primary stain, a step that makes some structures hold it (a mordant or heat), a decolourizer, and a counterstain.

WORKED EXAMPLE

Question (6 marks). A smear contains *Staphylococcus aureus* and *E. coli*. Give the colour of each after every step of a correct Gram stain. Then predict the final colours if (a) the iodine step is skipped, (b) the alcohol is left on for 2 minutes, (c) the alcohol step is skipped.

AFTER STEP	*S. AUREUS*	*E. COLI*
Crystal violet	Purple	Purple
Gram's iodine	Purple	Purple
Alcohol	Purple	Colourless
Safranin	Purple (Gram-positive)	Pink (Gram-negative)

1. (a) No iodine: no CV–I complex forms, so the alcohol washes crystal violet out of both. After safranin both are pink, and *S. aureus* is wrongly read as Gram-negative.
2. (b) Over-decolourizing: given enough time, alcohol strips the complex even from Gram-positive walls. Many *S. aureus* cells turn pink: false Gram-negatives.
3. (c) No alcohol: nothing is removed, so both stay purple and *E. coli* is wrongly read as Gram-positive.

EXAM TRAPS

- Purple is positive, pink is negative. "P for purple and positive" saves marks.
- Decolourization is the critical step: too long gives false negatives, too short gives false positives.
- Iodine is a mordant, not a stain; safranin is the counterstain, not the primary stain.
- Old cultures can stain Gram-variable; always use an 18–24-hour culture.
- Without the counterstain, Gram-negative cells are colourless and easily missed.

CHECK YOURSELF

– REMEMBER **List the Gram-stain reagents in order and give the role of each.** 4 marks

Crystal violet (primary stain), Gram's iodine (mordant), 95% ethanol or acetone–alcohol (decolourizer), safranin (counterstain).

– REMEMBER **Name two basic dyes and two acidic dyes.** 2 marks

Basic: methylene blue, crystal violet, safranin, malachite green. Acidic: eosin, nigrosin, Congo red.

– UNDERSTAND **Why do basic dyes stain bacterial cells while acidic dyes stain the background?** 2 marks

The cell surface is negatively charged at near-neutral pH. It attracts the positive chromophore of a basic dye and repels the negative chromophore of an acidic dye.

– UNDERSTAND **Why is a smear heat-fixed, and when is heat-fixing avoided?** 2 marks

To kill the cells, stick them to the slide and let dyes in. It is avoided in negative staining and in capsule and flagella stains, where heat would shrink or damage the structures.

– APPLY **An 18-hour culture of *Bacillus subtilis*, a Gram-positive rod, appears pink after Gram staining. Name two likely errors.** 2 marks

Over-decolourizing (alcohol on too long), or a skipped or weak iodine step. Over-heating the smear, which damages walls, is a third possibility.

– ANALYZE ***Mycoplasma* has no cell wall. Predict how it behaves in the Gram stain and explain why the result does not classify it.** 3 marks

It cannot hold the CV–I complex without peptidoglycan, so it loses the dye and stains pink or faintly. The Gram reaction tests wall structure; with no wall, a pink result does not mean it has a Gram-negative wall.

BEYOND THE SYLLABUS

Hospitals read Gram stains of blood cultures, spinal fluid and sputum within minutes to guide the first choice of antibiotic. Automated slide stainers make the decolourizing step consistent, and image-analysis software is being trained to read Gram-stained slides. A quick check without a microscope is the KOH string test: Gram-negative cells mixed with 3% potassium hydroxide burst and make the drop stringy. Search "KOH string test" to read more. This is not for the exam.

1.9 Special stains: acid-fast, endospore, capsule, flagella

Special stains show what the Gram stain misses: the waxy-walled bacilli of tuberculosis and leprosy, heat-resistant endospores, slimy capsules, and flagella too thin to see. Each method either forces dye past a barrier (heat drives carbol fuchsin into waxy walls and malachite green into spore coats) or works around it (capsules are shown by darkening the background, flagella by thickening them with a mordant).

You need: dyes, mordants and decolourizers from Chapter 1.8.

Acid-fast staining (Ziehl–Neelsen)

Mycobacteria have a cell wall rich in mycolic acids, very long waxy fatty acids that keep most dyes out. Once stained, though, they hold the dye even when washed with acid: they are acid-fast. Franz Ziehl (1882) and Friedrich Neelsen (1883) developed the hot method still in use. India's TB programme specifies these reagents for sputum smears:

1. Flood the fixed smear with 1% carbol fuchsin (basic fuchsin with phenol). Heat gently until vapours rise, without boiling, and leave it for 5 minutes. Phenol and heat drive the dye into the waxy wall.
2. Decolourize with 25% sulphuric acid for 2–4 minutes, repeating if the smear is still red. (The classic alternative is acid-alcohol, 3% HCl in 95% ethanol.) Other cells lose the dye; acid-fast bacilli keep it.
3. Counterstain with 0.1% methylene blue for 30 seconds.

Acid-fast bacilli (AFB) appear as thin red rods; everything else, including pus cells and other bacteria, is blue. Weakly acid-fast organisms need weaker acid: 5% sulphuric acid for *Mycobacterium leprae*, and about 1% for *Nocardia* and the oocysts of the parasite *Cryptosporidium*. The Kinyoun cold method uses more concentrated phenol and dye instead of heat, and the auramine fluorescent method (Chapter 1.7) is faster to read. Sputum smears are graded by how many AFB are seen under oil immersion:

AFB SEEN	FIELDS EXAMINED	REPORT
None in 100 fields	100	Negative
1–9 in 100 fields	100	Scanty (record the number)
10–99 in 100 fields	100	1+

AFB SEEN	FIELDS EXAMINED	REPORT
1–10 per field	50	2+
More than 10 per field	20	3+

Endospore staining (Schaeffer–Fulton)

Endospores of *Bacillus* and *Clostridium* have tough, almost impermeable coats (structure in Unit 2). In a Gram stain they show as clear, unstained holes inside cells.

1. Cover the smear with malachite green and steam it over boiling water for about 5 minutes, adding dye so it never dries. Heat drives the dye into the spores.
2. Cool and rinse with water for about 30 seconds. Water is the decolourizer: malachite green binds loosely to vegetative cells and washes out, but stays locked inside spores.
3. Counterstain with safranin for 30–60 seconds.

Spores are green and vegetative cells pink-red. The position and shape of the spore help identification: central and not swelling the cell in *Bacillus*; oval and subterminal in *Clostridium botulinum*; round, terminal and bulging, like a drumstick, in *Clostridium tetani*. Unstained spores look bright and refractile under phase contrast (Chapter 1.6).

Capsule staining

A capsule is a layer of polysaccharide (polypeptide in *Bacillus anthracis*) outside the wall. It helps bacteria escape being swallowed by white blood cells, so capsulated strains are often more virulent. Capsules are mostly water, take up dyes poorly, and shrink or vanish when heat-fixed, so they are shown by contrast:

- **Negative staining.** Mix the culture with a drop of India ink or nigrosin, spread it thin and air-dry without heat. The background is dark and each capsule is a clear halo around its cell; a simple stain added afterwards colours the cell itself.
- **Anthony's method.** Crystal violet, then 20% copper sulphate, which acts as both decolourizer and counterstain: cells deep purple, capsules pale blue.

In hospitals, an India ink preparation of spinal fluid shows the large capsule of the yeast *Cryptococcus neoformans* in cryptococcal meningitis. *Klebsiella pneumoniae* and *Streptococcus pneumoniae* are well-known capsulated bacteria.

Flagella staining

Bacterial flagella are only about 20 nm thick, about ten times thinner than the resolution limit of the light microscope. Flagella stains such as Leifson's use a mordant (tannic acid) with a dye

(basic fuchsin) that builds up on each flagellum until it is thick enough to see. The method is delicate: use a young culture, handle cells gently because flagella break off, use a perfectly clean slide, and do not heat-fix. The stain shows the number and position of flagella, which helps identification:

- **Monotrichous:** one polar flagellum (*Vibrio cholerae*, *Pseudomonas aeruginosa*).
- **Lophotrichous:** a tuft at one pole (*Helicobacter pylori*).
- **Amphitrichous:** flagella at both poles (*Campylobacter jejuni*).
- **Peritrichous:** flagella all over the surface (*E. coli*, *Salmonella*, *Proteus*).
- **Atrichous:** no flagella, non-motile (*Shigella*, *Klebsiella*).

Motility itself is checked more simply with a hanging-drop preparation, motility agar (a semi-solid medium in which motile bacteria spread out from the stab line) or dark-field microscopy. Flagellar structure is in Unit 2.

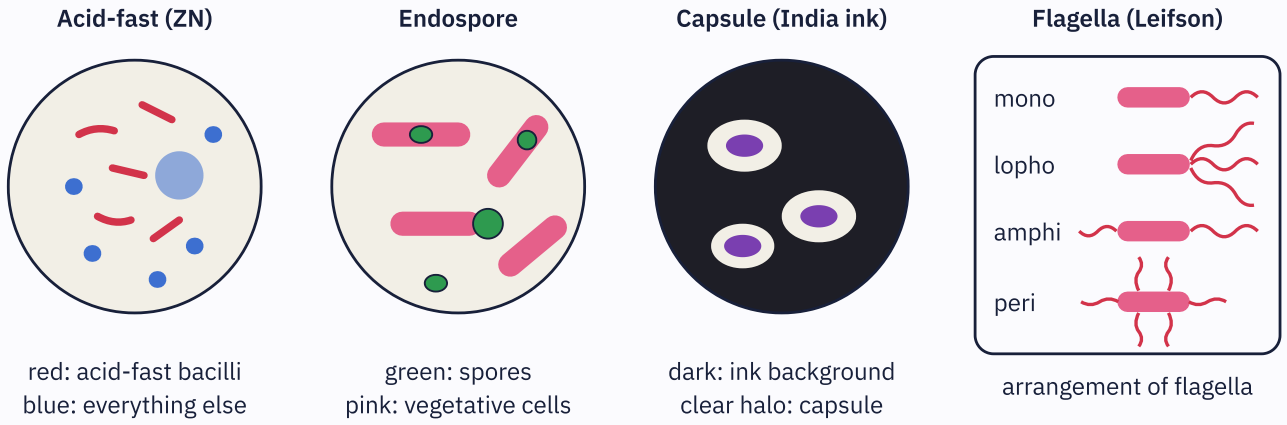


Figure 1.9.1 What each special stain looks like under oil immersion (sketches, not to scale). In the endospore field, notice the central spore (top), the drumstick-like terminal spore (middle) and a free spore (bottom). In the capsule field it is the background, not the capsule, that takes the colour.

WORKED EXAMPLE 1: GRADING A SPUTUM SMEAR

Question (4 marks). Using the grading table, report each Ziehl–Neelsen sputum smear: (a) 6 AFB in 100 oil-immersion fields; (b) 45 AFB in 100 fields; (c) an average of 4 AFB per field over 50 fields; (d) about 25 AFB per field in 20 fields.

1. (a) 1–9 AFB in 100 fields: positive, "scanty 6".
2. (b) 10–99 AFB in 100 fields: 1+.
3. (c) 1–10 AFB per field, 50 fields examined: 2+.
4. (d) More than 10 AFB per field, 20 fields examined: 3+.

Note that more fields are examined when fewer bacilli are present, so that a negative report means a real search, not a quick look.

WORKED EXAMPLE 2: SEEING A FLAGELLUM

Question (3 marks). A flagellum is 20 nm thick, and an oil objective resolves d = 0.22 µm. (a) By what factor must the stain thicken the flagellum before its width reaches d? (b) What does this mean for the image?

1. (a) 0.22 µm = 220 nm, and 220 ÷ 20 = 11. The coating must make the flagellum about eleven times thicker.
2. (b) The stained flagellum looks far thicker than it really is. Its number, position and wavy shape are reliable; its width is not.

EXAM TRAPS

- "Acid-fast" means the stained cell resists decolourization by acid; it does not mean the bacterium likes acid.
- In the endospore stain the decolourizer is water, and heat is applied with the primary stain, not the counterstain.
- Capsule stains use no heat-fixing. In India ink, the capsule is the clear zone, not the dark part.
- A flagella stain shows number and arrangement, not whether the cells were moving.

CHECK YOURSELF

– REMEMBER **Name the three reagents of the Ziehl–Neelsen stain and the colours of acid-fast and non-acid-fast cells.** 3 marks

Carbol fuchsin (with heat), 25% sulphuric acid or acid-alcohol, methylene blue. Acid-fast cells red, others blue.

– REMEMBER **Classify by flagella: *Vibrio cholerae*, *E. coli*, *Shigella*.** 2 marks

Monotrichous, peritrichous, atrichous.

– UNDERSTAND **Why is heat used in both the Ziehl–Neelsen and the Schaeffer–Fulton methods?** 2 marks

Both targets (the waxy mycobacterial wall and the spore coat) resist dyes; heat drives the primary stain in, after which it is held against the decolourizer.

– UNDERSTAND **Why does a capsule appear as a clear halo in an India ink preparation?** 2 marks

The ink particles cannot enter the capsule, so they darken only the background; the capsule, mostly water and unstained, stays clear around the cell.

– APPLY **A smear shows an average of 3 AFB per field over 50 fields. Another shows 8 AFB in 100 fields. Report both.** 2 marks

2+; scanty 8.

– ANALYZE **A Gram stain of a soil isolate shows Gram-positive rods with clear oval areas in the middle of some cells. What are the clear areas, how would you confirm it, and which genus is likely if the isolate grows in air?** 4 marks

Endospores, which resist the Gram dyes. Confirm with the Schaeffer–Fulton stain (green spores in pink cells) or phase contrast. An aerobic spore-forming rod is likely *Bacillus*; *Clostridium* grows only without oxygen.

BEYOND THE SYLLABUS

Smear microscopy detects TB only when sputum holds thousands of bacilli per millilitre, so India's TB programme also uses rapid molecular tests such as GeneXpert and the Indian-made Truenat, which detect *M. tuberculosis* DNA and rifampicin resistance within hours. Search "Truenat TB test" or "LED fluorescence microscopy TB" to read more. This is not for the exam.

Unit summary

TERM	MEANING OR FORMULA	CHAPTER
Biogenesis	Living cells arise only from living cells (Pasteur's swan-neck flasks, 1861)	1.1
Koch's postulates	Microbe in every case; grown in pure culture; culture causes the disease; microbe re-isolated	1.1
Prokaryote vs eukaryote	No nucleus, 70S ribosomes vs nucleus, organelles, 80S ribosomes	1.2
Surface-to-volume ratio	A/V = 3/r for a sphere of radius r	1.2
Diffusion time	$t \approx x^2/(2D)$	1.2
Three domains	Bacteria, Archaea, Eukarya (Woese, from rRNA sequences)	1.4
Similarity coefficients	S_SM = (a + d)/(a + b + c + d); S_J = a/(a + b + c)	1.4
IMViC	Indole, methyl red, Voges–Proskauer, citrate; *E. coli* + + − −	1.5
G + C from melting point	mol% G + C = $(T_m - 69.3) / 0.41$	1.5
Same-species rules of thumb	16S identity ≥ 98.7%; ANI ≥ 95–96%; DNA–DNA hybridization ≥ 70%	1.5
Total magnification	Objective × eyepiece (100 × 10 = 1000×)	1.6
Resolution	$d = 0.5 \lambda / NA$, $NA = n \sin \theta$; about 0.2 µm for light	1.6
Useful magnification	About 500–1000 × NA	1.6
Photon energy	E (eV) ≈ 1240 / λ (nm); emission always at longer λ	1.7
Electron wavelength	$\lambda = h / \sqrt{(2meV)}$; 0.0037 nm at 100 kV	1.7
Gram stain	Crystal violet → iodine → alcohol → safranin; positive purple, negative pink	1.8
Ziehl–Neelsen	Hot carbol fuchsin → 25% H_2SO_4 → methylene blue; acid-fast bacilli red	1.9

TERM	MEANING OR FORMULA	CHAPTER
Schaeffer–Fulton	Steamed malachite green → water → safranin; spores green, cells pink	1.9

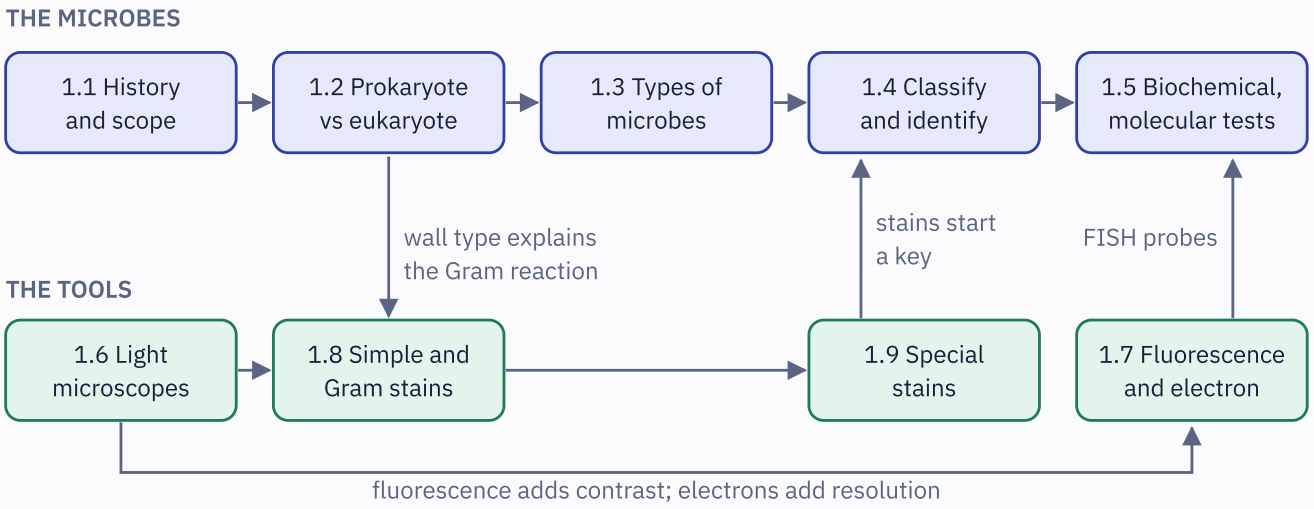


Figure U1.1 How the chapters connect. The top row builds the picture of the microbial world; the bottom row gives the tools for seeing it. The vertical arrows are where the two meet: cell-wall structure explains the Gram stain, stains are the first branch of every identification key, and fluorescent probes let microscopes identify microbes that cannot be cultured.

1. Microbiology rests on three answers: microbes exist (Leeuwenhoek), they arise only from other microbes (Pasteur), and specific microbes cause specific diseases (Koch).
2. Prokaryotes (bacteria and archaea) have no nucleus or membrane-bound organelles and have 70S ribosomes; eukaryotes (fungi, algae, protozoa) have both and 80S ribosomes. These differences are what selective antibiotics exploit.
3. Microbes range from about 20 nm (small viruses) to hundreds of micrometres (large protozoa), and most of them have never been grown in a laboratory.
4. Taxonomy is classification, nomenclature and identification. Woese's rRNA tree gives three domains, with archaea closer to eukaryotes than to bacteria.
5. Identification combines stains, biochemical tests (catalase, oxidase, IMViC, API strips), chemical fingerprints (MALDI-TOF) and gene sequences (16S rRNA, whole genomes).
6. Resolution, not magnification, limits what a microscope shows: $d = 0.5\,\lambda / NA$ gives about 0.2 µm for light, and immersion oil raises NA.
7. Dark-field and phase contrast show living, unstained cells; fluorescence adds colour and specificity; electron microscopes resolve detail about a thousand times finer than light microscopes.
8. The Gram stain sorts bacteria by wall structure, and decolourization is its critical step.

9. Special stains use heat (acid-fast, endospore), negative staining (capsule) or a mordant (flagella) to show what the Gram stain misses.

Exam question bank

Remember and understand

- REMEMBER **Trace the history of microbiology from Leeuwenhoek to Koch, and explain how the spontaneous-generation debate was settled.** 12 marks

  Leeuwenhoek's animalcules (1676); Redi, Needham, Spallanzani; Pasteur's swan-neck flasks (1861) with a labelled sketch and the broken-neck control; Tyndall and Cohn on endospores; fermentation and pasteurization; Semmelweis and Lister; Koch's anthrax work (1876), pure culture on agar, tuberculosis (1882), cholera in Calcutta (1884) and the four postulates with two limitations.

- UNDERSTAND **Compare prokaryotic and eukaryotic cells in a table of at least ten features, with labelled diagrams.** 10 marks

  Size; nucleus vs nucleoid; chromosomes and histones; 70S vs 80S ribosomes; organelles; site of respiration; wall chemistry; sterols; flagella structure; division; gene expression. Note archaea and the endosymbiont theory; end with selective toxicity (penicillin, 70S-ribosome antibiotics).

- UNDERSTAND **Explain the scope of microbiology, its branches and the objectives of studying it.** 8 marks

  Branches by organism (bacteriology, virology, mycology, phycology, protozoology) and by application (medical, food, industrial, agricultural, environmental, genetic) with one Indian example each; the four objectives: understand, control, use, model organisms.

- UNDERSTAND **Describe the principle, construction and uses of the phase-contrast microscope.** 10 marks

  Phase vs brightness; annular diaphragm; phase plate and ring; diffracted light about λ/4 behind, ring adds λ/4, destructive interference at λ/2; dark cell on grey field; halo artefact; uses (live cells, division, endospores, protozoa); comparison with bright-field; labelled diagram.

– UNDERSTAND **Explain the three-domain system. Why did Woese use rRNA, and how does the system differ from Whittaker's five kingdoms?** 8 marks

Five kingdoms (1969) and their basis; 16S/18S rRNA, its universality and conserved plus variable regions; Bacteria, Archaea, Eukarya with one distinguishing feature each; the tree with archaea and eukaryotes as sister groups; domains sit above kingdoms.

Apply

– APPLY **Define numerical aperture and resolving power. Calculate d for a 40× objective (NA 0.65) and a 100× oil objective (NA 1.25) at 550 nm, and the useful magnification of each.** 8 marks

NA = n sin θ; d = 0.5 λ / NA; 423 nm and 220 nm; useful magnification 325–650× and 625–1250×; role of oil (n = 1.515); empty magnification.

– APPLY **Describe the Gram stain: procedure, mechanism, and the result of skipping iodine, over-decolourizing and skipping the counterstain.** 10 marks

Smear and fixing; the four reagents with times; colour table for both cell types after each step; wall-based mechanism (thick peptidoglycan vs outer membrane); error outcomes (both pink; false negatives; Gram-negatives invisible); controls and culture age.

– APPLY **Describe the Ziehl–Neelsen method for sputum and grade smears with 7 AFB per 100 fields, 2 AFB per field over 50 fields and 15 AFB per field over 20 fields.** 8 marks

Mycolic acids; 1% carbol fuchsin with heat for 5 minutes, 25% sulphuric acid for 2–4 minutes, 0.1% methylene blue for 30 seconds; red bacilli on blue; grades: scanty 7, 2+, 3+; Kinyoun and fluorescent alternatives.

– APPLY **A Gram-negative rod is isolated from a patient's urine. Plan its identification from the first stain to a final name, saying what each step tells you.** 12 marks

Gram stain and oil-immersion morphology (Chapters 1.6, 1.8); oxidase and catalase; IMViC or an API 20E profile (Chapter 1.5); a dichotomous key and Bergey's Manual (Chapter 1.4); MALDI-TOF where available; 16S sequencing if the result is doubtful; a note that stains give the first branch of the key.

– APPLY **Four strains were scored on 20 tests. Explain how to compute the simple matching and Jaccard coefficients for each pair and build a dendrogram from them.** 8 marks

Count a, b, c, d for each pair; S_SM and S_J formulas; a similarity matrix; join the most similar pair first; average linkage for the next joins; draw the dendrogram with a similarity scale; state which coefficient and why.

Analyze and evaluate

– ANALYZE **Compare bright-field, dark-field, phase-contrast, fluorescence, TEM and SEM microscopes, and choose one for each: live spirochaetes, virus structure, screening many TB smears, the surface of a biofilm, endospores in a live culture.** 12 marks

Table of principle, illumination, specimen, resolution and image for all six. Choices: dark-field; TEM with negative staining; auramine fluorescence; SEM; phase contrast. Justify each with one line.

– EVALUATE **"Resolution, not magnification, decides what a microscope can show." Evaluate this statement with calculations.** 8 marks

Define both; d = 0.5 λ / NA; show that a 20× eyepiece doubles size but not detail; useful magnification 500–1000 × NA; dark-field detects sub-resolution objects without resolving them; electron wavelength and the TEM's 0.2 nm.

– EVALUATE **Evaluate 16S rRNA gene sequencing against biochemical identification and genome-based methods.** 10 marks

16S: universal primers, works on unculturable microbes, large databases; limits: cannot separate close species such as *E. coli* and *Shigella*, thresholds are rules of thumb. Biochemical: cheap, shows phenotype, but slow and needs pure culture. Genomes: ANI and typing give the finest resolution at higher cost. Conclude with the polyphasic approach.

– ANALYZE **Koch's postulates were written for culturable bacteria. Discuss their limitations with examples and how newer methods extend them.** 8 marks

Healthy carriers; unculturable agents (*M. leprae*, *T. pallidum*); viruses that need host cells; diseases caused by several microbes; no animal model; ethics. Extensions: molecular Koch's postulates (genes rather than organisms), DNA detection in tissue, metagenomics.

Glossary

Abiogenesis	The old idea that life arises from non-living matter; also called spontaneous generation.
Acid-fast	Holding carbol fuchsin even when washed with acid, because of a waxy, mycolic-acid-rich wall.
ANI	Average nucleotide identity between two genomes; 95–96% or more suggests one species.
Biogenesis	The principle that living cells arise only from living cells.
Capsule	A loose layer of polysaccharide or polypeptide outside the cell wall.
Chromophore	The coloured, charged part of a dye molecule.
Condenser	The lens system below the stage that focuses light on the specimen.
Counterstain	A second dye of contrasting colour that stains cells decolourized in a differential stain.
Decolourizer	A reagent, such as alcohol or acid, that removes dye from some cells but not others.
Dichroic mirror	A mirror that reflects short wavelengths and transmits longer ones, used in fluorescence microscopes.
Differential stain	A stain using two or more dyes that divides bacteria into groups, such as the Gram stain.
Endospore	A tough, dormant structure formed inside some bacteria that survives heat and drying.
Fluorochrome	A dye that absorbs light of one wavelength and emits light of a longer wavelength.
Koch's postulates	Four criteria for proving that a particular microbe causes a particular disease.
Mordant	A substance that increases the binding of a dye or traps it in the cell, such as Gram's iodine.
Negative staining	Staining the background with an acidic dye or ink so that cells or capsules show up unstained.
Nucleoid	The region of a prokaryotic cell that holds the chromosome, with no membrane around it.
Numerical aperture	NA = n sin θ; a measure of how wide a cone of light an objective collects.
Phase contrast	Microscopy that turns differences in refractive index into differences in brightness.
Polyphasic taxonomy	Classification that combines phenotypic, chemical and genetic data.

Resolution	The smallest distance between two points that can still be seen as separate.
Serovar	Strains of a species that differ in their surface antigens.
Stokes shift	The difference between the peak excitation and emission wavelengths of a fluorochrome.
Strain	A pure-culture line within a species, descended from one isolate.
Svedberg unit (S)	A measure of how fast a particle sediments in a centrifuge; ribosomes are 70S or 80S.
Taxonomy	The science of classification, nomenclature and identification.
Type strain	The reference culture that carries a species name, kept in public culture collections.

Sources and further reading

- Willey, Sandman and Wood, *Prescott's Microbiology*, McGraw-Hill: history, cell structure, microscopy, staining and taxonomy (from memory; check the edition you are prescribed).
- Tortora, Funke and Case, *Microbiology: An Introduction*, Pearson (from memory; check).
- Madigan and colleagues, *Brock Biology of Microorganisms*, Pearson (from memory; check).
- Pelczar, Chan and Krieg, *Microbiology*, Tata McGraw-Hill, 5th edition (from memory; check).
- *Ananthanarayan and Paniker's Textbook of Microbiology*, Universities Press: staining methods as used in Indian medical laboratories (from memory; check).
- Dubey and Maheshwari, *A Textbook of Microbiology*, S. Chand (from memory; check).
- Whitman, Coleman and Wiebe (1998), "Prokaryotes: the unseen majority", *Proceedings of the National Academy of Sciences*: the source of the 4–6 × 10^{30} estimate in Chapter 1.3 (from memory; check).
- OpenStax, *Microbiology* (2016), Chapter 2, "How We See the Invisible World", read in the SUNY and Lumen Learning edition: courses.lumenlearning.com/suny-microbiology. Used for light-microscope magnification limits and TEM section thickness.
- Central TB Division, Government of India, Technical and Operational Guidelines, Annexure 1 (Ziehl–Neelsen sputum smear method and grading): tbcindia.mohfw.gov.in.

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