Microbiology

Unit 1 · Lesson 4 · Staining bacteria

The notes for Lesson 4 of Unit 1: chapters 1.8 to 1.9 of the unit book, with their figures, worked examples, exam traps and quizzes, in one file. The full unit book, with every chapter, the exam question bank and the glossary, is linked at the end.

Lesson 4 of 4 | 2 chapters | Chapters 1.8 to 1.9

About these notes

These are the notes for **Lesson 4 · Staining bacteria** of the University free n easy video lessons on Microbiology Unit 1, Foundations: Microbial World and Microscopy. They are chapters 1.8 to 1.9 of the unit book, unchanged, so a page here is the same page in the full book.

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.

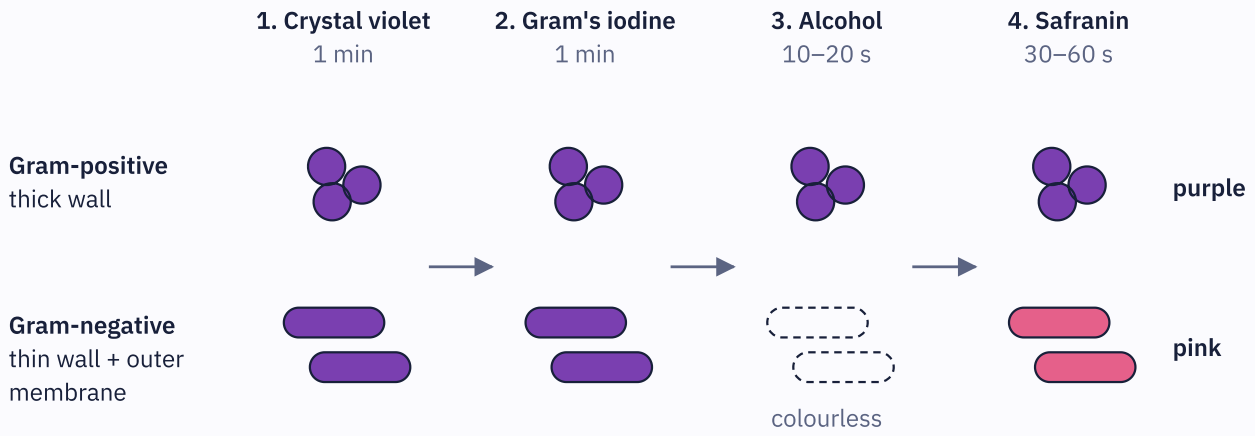


Figure 1.8.1 The colour of each cell type after each step. Notice that the two types look identical until step 3: the alcohol is the only step that tells them apart, and safranin only makes the decolourized Gram-negative cells visible again.

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.

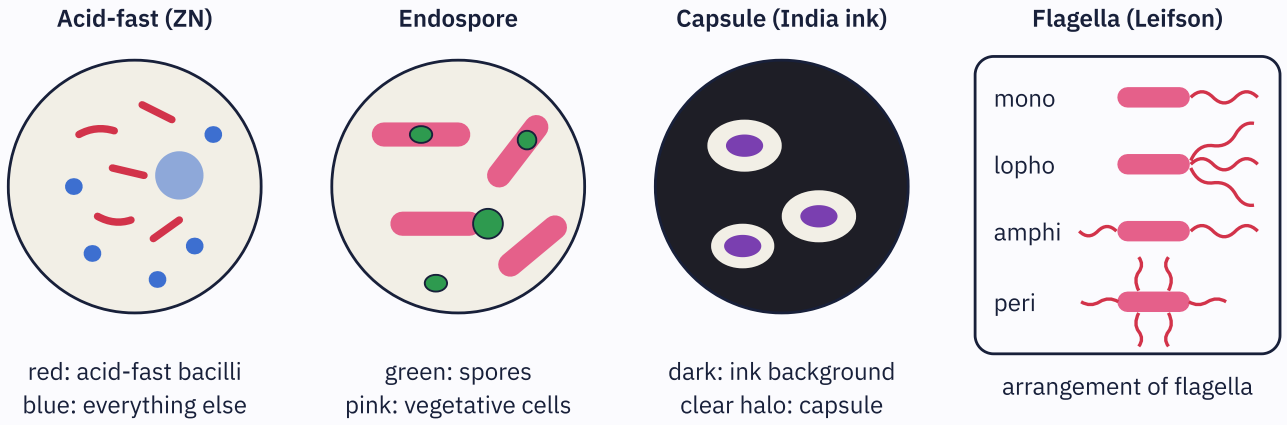


Figure 1.9.1 What each special stain looks like under oil immersion (sketches, not to scale). In the endospore field, notice the central spore (top), the drumstick-like terminal spore (middle) and a free spore (bottom). In the capsule field it is the background, not the capsule, that takes the colour.

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.

Key terms of this lesson

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

Glossary (terms used in this lesson)

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.

Capsule A loose layer of polysaccharide or polypeptide outside the cell wall.

Chromophore The coloured, charged part of a dye molecule.

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.

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.

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.

Phase contrast Microscopy that turns differences in refractive index into differences in brightness.

Resolution	The smallest distance between two points that can still be seen as separate.
Strain	A pure-culture line within a species, descended from one isolate.

The full unit book

Every chapter of Unit 1, the unit summary, the exam question bank with model answers and the full glossary: Microbiology · Unit 1 · Foundations: Microbial World and Microscopy (one file, works offline).

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.

Syllabus: the University free n easy universal syllabus, built from top universities' syllabi.

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