Microbiology

Unit 1 · Lesson 3 · Microscopes: light to electrons

The notes for Lesson 3 of Unit 1: chapters 1.6 to 1.7 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 3 of 4 | 2 chapters | Chapters 1.6 to 1.7

About these notes

These are the notes for **Lesson 3 · Microscopes: light to electrons** of the University free n easy video lessons on Microbiology Unit 1, Foundations: Microbial World and Microscopy. They are chapters 1.6 to 1.7 of the unit book, unchanged, so a page here is the same page in the full book.

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:

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

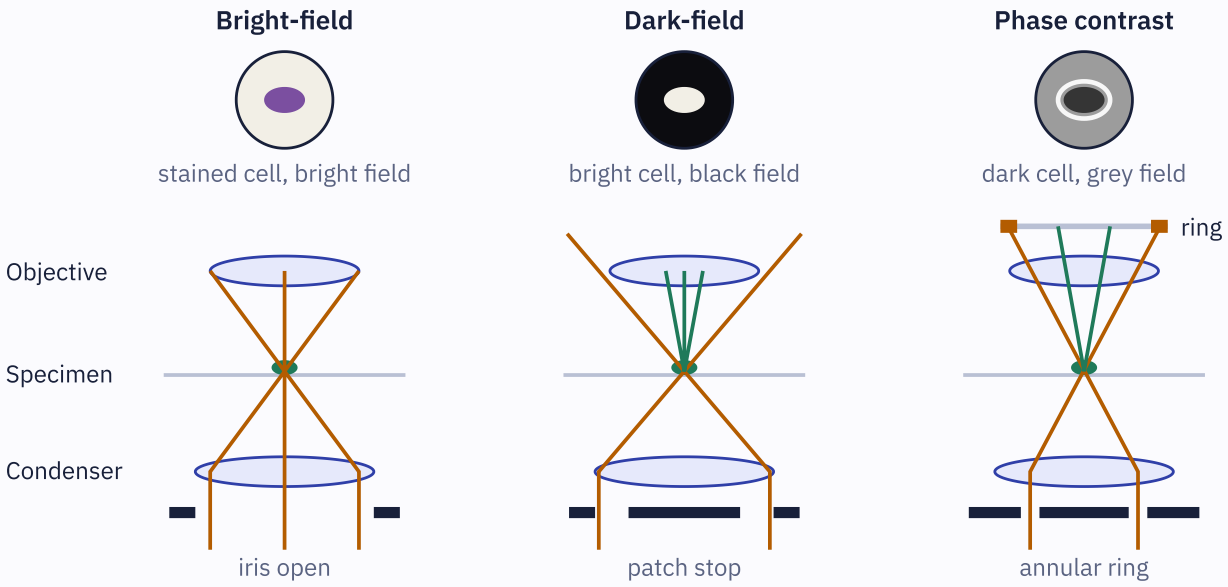


Figure 1.6.1 Light (orange) comes up from the lamp. Bright-field: the whole cone enters the objective. Dark-field: the patch stop makes a hollow cone that misses the objective, so only light scattered by the specimen (green) is collected. Phase contrast: the hollow cone from the annular ring lands on 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.

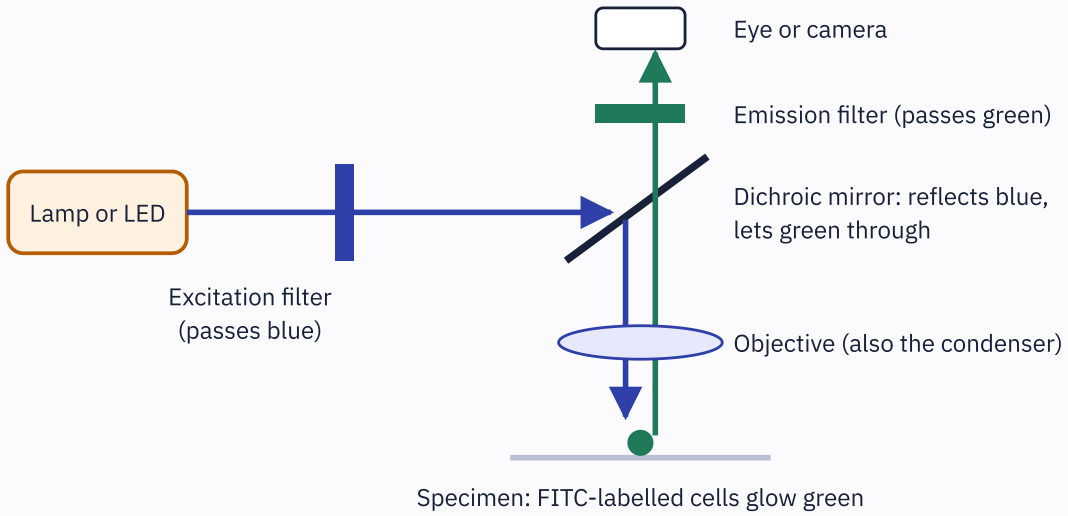


Figure 1.7.1 Blue excitation light (left) is reflected down onto the specimen; green emitted light travels up through the mirror and the emission filter. Notice that the two filters and the mirror together keep the bright exciting light away from the eye, which is why fluorescing cells appear on a black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

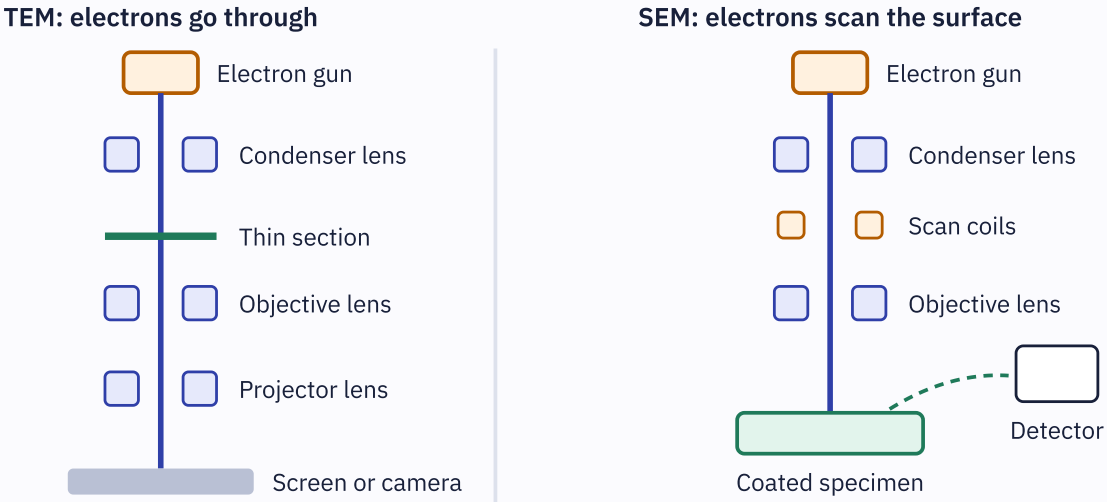


Figure 1.7.2 Both columns are under vacuum and use electromagnetic lenses (small squares). In the TEM the image is formed by electrons that pass through a thin section; in the SEM the scan coils (orange) sweep the beam over a coated specimen, and a detector collects the secondary electrons knocked out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

Key terms of this lesson

TERM	MEANING OR FORMULA	CHAPTER
Total magnification	Objective × eyepiece (100 × 10 = 1000×)	1.6
Resolution	d = 0.5 λ / NA, NA = n sin θ; 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	λ = h / √(2meV); 0.0037 nm at 100 kV	1.7

Glossary (terms used in this lesson)

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

Condenser The lens system below the stage that focuses light on the specimen.

Dichroic mirror A mirror that reflects short wavelengths and transmits longer ones, used in fluorescence microscopes.

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.

Negative staining Staining the background with an acidic dye or ink so that cells or capsules show up unstained.

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.

Resolution The smallest distance between two points that can still be seen as separate.

Stokes shift The difference between the peak excitation and emission wavelengths of a fluorochrome.

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.

Built for the University free n easy YouTube channel from the universal syllabus of Microbiology (Oct 2026). Written with top universities' syllabi and standard textbooks; check a formula against your prescribed book before an exam, and report mistakes on the channel.