Cell Biology

Unit 6 · Specialized Cells and Cell Study Methods

How do a neuron, a muscle fibre and a β cell do such different jobs with the same parts, and how do biologists see and take apart cells to find out? After this unit you can explain the action potential, muscle contraction, phototransduction and insulin release, choose the right microscope for a question, calculate resolution, centrifuge settings and cell counts, and design an experiment that finds where a protein lives.

Taught at 7 of 10 | Usually Sem 3 | 8 chapters

About this unit

This unit has two halves. The first looks at specialised cells, where the membranes, channels, motors and signals of Units 2 to 5 are put to work: nerve cells that fire action potentials (Chapter 6.1), muscle cells that contract (6.2), and the rods, cones and pancreatic cells that sense light and glucose (6.3). The second half is the toolkit used to study any cell: light and fluorescence microscopes (6.4), electron and confocal microscopes (6.5), cell fractionation to isolate organelles (6.6), flow cytometry to measure thousands of single cells (6.7), and immunostaining to find where a protein sits (6.8). Each method builds on the one before it.

Chapters

1. Nerve cells: structure and function ADVANCED
2. Muscle cells: types and function ADVANCED
3. Cells of vision and pancreatic cells ADVANCED
4. Light and fluorescence microscopy
5. Electron and confocal microscopy ADVANCED
6. Cell fractionation and isolation of organelles
7. Flow cytometry ADVANCED
8. Immunostaining and protein localization

How to use this book

- Read the lead paragraph and the figure of each chapter first; they carry the one idea.
- Cover the solution of the worked example and do it yourself with a pencil, then compare step by step.
- Answer each "Check yourself" question in your head or on paper before you open it.

6.1 Nerve cells: structure and function

A nerve cell, or neuron, is a cell built to carry a message fast and far: from your toe to your spinal cord in a few hundredths of a second. It sends an electrical signal along a long, thin axon and then a chemical signal across a small gap, the synapse. Other universities meet neurons in physiology courses.

> You need: ion channels and the Na^+/K^+ pump (Unit 2, Ion channels: ligand-gated and voltage-gated; Active transport) and exocytosis (Unit 2, Endocytosis and exocytosis).

Specialised cells: one genome, many jobs

Every cell in your body carries the same DNA, yet a neuron, a muscle fibre and a pancreatic β cell work nothing alike. Specialisation (differentiation) comes from which genes are switched on: each cell type makes its own proteins and builds its shape around one job. Chapters 6.1 to 6.3 look at four such cells.

Parts of a neuron

- **Dendrites**: short branches that receive signals from other cells.
- **Cell body (soma)**: holds the nucleus and most ribosomes; its clumps of rough ER are called Nissl bodies.
- **Axon hillock and initial segment**: where the axon leaves the soma. Voltage-gated Na^+ channels are densest here, so the action potential starts here.
- **Axon**: one long process, up to about a metre in human leg nerves, that carries the signal away from the soma.
- **Myelin sheath**: layers of membrane wrapped round the axon by Schwann cells (peripheral nerves) or oligodendrocytes (brain and spinal cord). The bare gaps between wraps are the **nodes of Ranvier**.
- **Axon terminals**: end in synapses, where vesicles of neurotransmitter wait.

By shape, neurons are multipolar (many dendrites, one axon; motor neurons), bipolar (one dendrite, one axon; retina) or pseudounipolar (one process that splits in two; sensory neurons).

Resting potential and the action potential

At rest the inside of a neuron is negative, about −70 mV. The Na^+/K^+-ATPase pumps 3 Na^+ out and 2 K^+ in per ATP, so K^+ is high inside and Na^+ high outside. The resting membrane is leaky mainly to K^+, so K^+ flows out until the negative charge left behind holds it back. The voltage at which one ion is in balance is its equilibrium potential, given by the Nernst equation:

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E = (RT / zF) · ln(C_out / C_in)
At 37 °C and z = +1:  E = 61.5 mV × log10(C_out / C_in)
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Here R is the gas constant (8.314 J mol^{-1} K^{-1}), T the temperature in kelvin, z the ion's charge, F the Faraday constant (96,485 C mol^{-1}) and C the ion concentration outside and inside.

1. A stimulus depolarises the membrane to threshold, about −55 mV.
2. Voltage-gated Na^+ channels open, Na^+ rushes in, and the inside swings to about +30 mV.
3. Within about a millisecond the Na^+ channels inactivate and voltage-gated K^+ channels open. K^+ leaves and the membrane repolarises.
4. The K^+ channels close slowly, so the voltage briefly undershoots below rest (after-hyperpolarisation) before leak channels and the pump restore −70 mV.

While the Na^+ channels are inactivated (the refractory period) they cannot reopen, so the spike moves only forward. The action potential is all-or-none: a stronger stimulus gives more spikes per second, not a taller spike. In an unmyelinated axon the spike creeps along at about 1 m/s. In a myelinated axon it jumps from node to node (saltatory conduction), up to about 100 m/s, and only the nodes exchange ions, which saves ATP.

The synapse

At a chemical synapse the spike opens voltage-gated Ca^{2+} channels in the terminal. Ca^{2+} entry makes vesicles fuse with the membrane, and transmitter (acetylcholine, glutamate, GABA) crosses a cleft of about 20 nm. It binds ligand-gated channels on the next cell, which either excite it (cations in) or inhibit it (Cl^- in). The transmitter is then removed: acetylcholinesterase splits acetylcholine, and other transmitters are pumped back. Electrical synapses use gap junctions (Unit 5, Cell junctions and cell adhesion) and pass current directly.

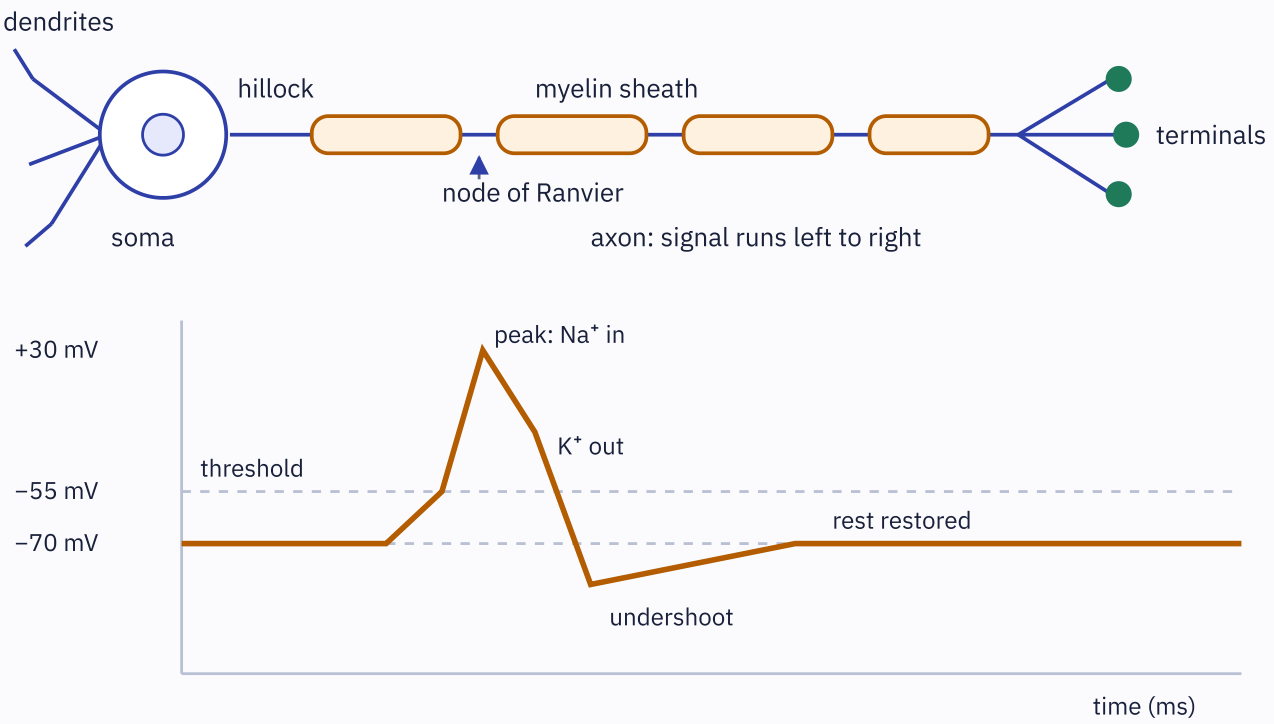


Figure 6.1.1 Top: the parts of a myelinated neuron; the spike starts at the hillock and jumps between the nodes. Bottom: one action potential, about 2 ms long. Notice the threshold line: below it nothing happens, above it the full spike always fires.

WORKED EXAMPLE

Question (6 marks). A mammalian neuron has K^+ = 140 mM inside and 5 mM outside, Na^+ = 15 mM inside and 145 mM outside, at 37 °C. Find E_K and E_{Na}. Explain why the resting potential (−70 mV) lies close to E_K and the spike peak moves towards E_{Na}.

1. $E_K = 61.5 \times \log_{10}(5/140) = 61.5 \times \log_{10}(0.0357) = 61.5 \times (-1.447) =$ **−89 mV**.
2. $E_{Na} = 61.5 \times \log_{10}(145/15) = 61.5 \times \log_{10}(9.67) = 61.5 \times 0.985 =$ **+61 mV**.
3. At rest the membrane is far more permeable to K^+ than to Na^+, so the voltage sits near E_K. A small Na^+ leak pulls it up from −89 to about −70 mV.
4. When voltage-gated Na^+ channels open, Na^+ permeability briefly dominates and the voltage heads towards E_{Na}. It peaks below +61 mV (about +30 mV) because Na^+ channels inactivate and K^+ channels open first.

EXAM TRAPS

- The pump moves 3 Na^+ out and 2 K^+ in, not the reverse, and not 1:1.
- Schwann cells myelinate peripheral axons; oligodendrocytes myelinate in the brain and spinal cord. Examiners swap them.
- A stronger stimulus does not make a bigger spike; it makes more spikes per second.
- Repolarisation is K^+ leaving the cell, not Na^+ being pumped out. The pump is far too slow to shape one spike.

CHECK YOURSELF

– REMEMBER **Name the cells that form myelin in the PNS and in the CNS.** 2 marks

Schwann cells in the peripheral nervous system; oligodendrocytes in the central nervous system.

– UNDERSTAND **Why does an action potential travel only away from the soma?** 3 marks

Behind the spike the Na^+ channels are inactivated (refractory) and cannot reopen, so only the membrane ahead can be pushed to threshold.

– UNDERSTAND **Explain the role of Ca^{2+} at a chemical synapse.** 3 marks

The spike opens voltage-gated Ca^{2+} channels in the terminal; Ca^{2+} entry triggers synaptic vesicles to fuse with the membrane and release transmitter by exocytosis.

– APPLY **Outside K^+ rises from 5 to 10 mM (inside 140 mM). Find the new E_K and say what happens to the resting potential.** 4 marks

$E_K = 61.5 \times \log_{10}(10/140) = 61.5 \times (-1.146) =$ **−70 mV**. E_K becomes less negative, so the cell depolarises, sits nearer threshold and becomes more excitable; this is why high blood K^+ disturbs heart rhythm.

– ANALYZE **Myelin raises conduction speed and saves energy. Explain both from the same mechanism.** 5 marks

Myelin insulates the internode, so current spreads quickly inside the axon to the next node instead of leaking out. The spike is regenerated only at nodes: it jumps (faster), and ions cross the membrane only at nodes, so the pump has far fewer ions to move back (less ATP).

BEYOND THE SYLLABUS

Not for the exam. Optogenetics puts light-gated channels into chosen neurons so a researcher can switch them on with a flash of blue light. Brain-computer interfaces read spikes from electrode arrays to move a cursor or a robotic arm. Search next: "patch clamp" and "optogenetics channelrhodopsin".

6.2 Muscle cells: types and function

A muscle cell turns chemical energy (ATP) into pulling force. Myosin motors walk along actin filaments, and because thousands of them pull at once, the whole cell shortens. Your body has three kinds of muscle cell, skeletal, cardiac and smooth, built on the same actin–myosin engine but switched on in different ways.

You need: actin filaments and myosin motors (Unit 3, Cytoskeleton; Molecular motors and centrioles) and the action potential (Chapter 6.1).

Three types of muscle cell

FEATURE	SKELETAL	CARDIAC	SMOOTH
Shape	Long cylindrical fibre, many nuclei at the edge	Short, branched, usually one central nucleus	Spindle-shaped, one central nucleus
Striations	Yes (sarcomeres)	Yes (sarcomeres)	No
Control	Voluntary, by motor neurons	Involuntary, own pacemaker	Involuntary, nerves and hormones
Cell links	Each fibre separate	Intercalated discs with gap junctions	Gap junctions in many tissues
Ca^{2+} switch	Troponin on thin filament	Troponin on thin filament	Calmodulin and myosin light-chain kinase
Where	Limbs, trunk, face	Heart wall	Gut, blood vessels, uterus, bladder

A skeletal muscle fibre is a single huge cell formed when many myoblasts fuse during development; that is why it has many nuclei. Inside, it is packed with myofibrils, each a chain of

repeating units called sarcomeres.

The sarcomere and the sliding filament model

A sarcomere runs from one Z line (Z disc) to the next. Thin filaments of actin are anchored at the Z lines and point inwards; thick filaments of myosin sit in the middle, held at the M line. Under the microscope this gives bands:

- **A band**: the length of the thick filaments (dark, anisotropic). It does not change during contraction.
- **I band**: the part with thin filaments only (light, isotropic). It shortens.
- **H zone**: the middle of the A band, with thick filaments only. It shortens and can vanish.

The sliding filament model (A. F. Huxley and R. Niedergerke; H. E. Huxley and J. Hanson, both 1954) says the filaments do not shorten; they slide past each other, pulling the Z lines closer.

Cross-bridge cycle and excitation–contraction coupling

1. A motor neuron releases acetylcholine at the neuromuscular junction; the fibre fires an action potential.
2. The spike runs down T-tubules (inward folds of the plasma membrane). A voltage sensor there (the dihydropyridine receptor) opens ryanodine receptors in the sarcoplasmic reticulum (SR), and Ca^{2+} floods the cytosol.
3. Ca^{2+} binds troponin C; troponin shifts tropomyosin off the myosin-binding sites on actin.
4. A myosin head carrying ADP + Pi binds actin. Releasing Pi triggers the power stroke, pulling the thin filament towards the M line; ADP leaves.
5. A fresh ATP binds and the head lets go of actin. Hydrolysis of that ATP re-cocks the head for the next cycle.
6. When nerve firing stops, the SR Ca^{2+} pump (SERCA) takes Ca^{2+} back, tropomyosin covers the sites and the muscle relaxes.

ATP is needed both to detach myosin and to pump Ca^{2+} back. After death ATP runs out, heads stay locked on actin, and the muscles go stiff: rigor mortis. In smooth muscle, Ca^{2+} binds calmodulin, which activates myosin light-chain kinase; the phosphorylated myosin then binds actin. Cardiac cells add a Ca^{2+} entry step from outside and pass the spike to neighbours through gap junctions, so the heart contracts as one unit.

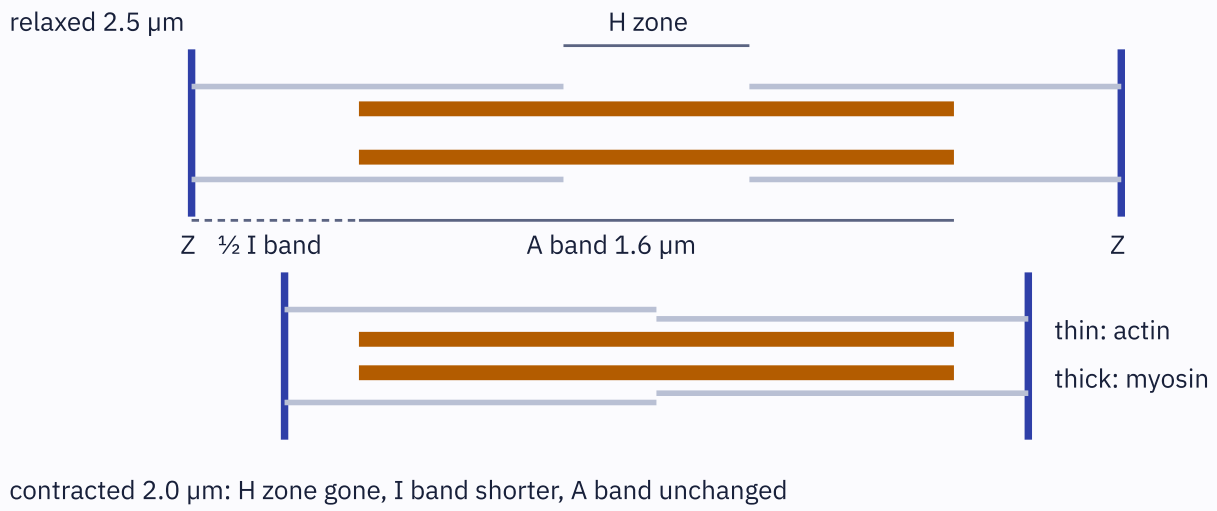


Figure 6.2.1 One sarcomere before and after contraction, drawn to scale with the lengths of the worked example (200 units = 1 µm). The thick filaments (orange) keep their length, so the A band is the same; the thin filaments slide inwards, so the I band and H zone shrink and the Z lines move closer.

WORKED EXAMPLE

Question (5 marks). In a frog muscle, thick filaments are 1.6 µm long and each thin filament (from a Z line) is 1.0 µm long. A sarcomere shortens from 2.5 µm to 2.0 µm. Find the A band, I band and H zone before and after.

1. A band = thick filament length = **1.6 µm** both times.
2. I band = sarcomere − A band. Before: 2.5 − 1.6 = **0.9 µm**. After: 2.0 − 1.6 = **0.4 µm**.
3. H zone = sarcomere − 2 × thin filament length. Before: 2.5 − 2.0 = **0.5 µm**. After: 2.0 − 2.0 = **0 µm**; the thin filaments now meet at the centre.
4. Check: the I band lost 0.5 µm and the H zone lost 0.5 µm, equal to the 0.5 µm shortening, as sliding predicts.

EXAM TRAPS

- The A band never changes length. Only the I band and H zone shorten.
- ATP binding makes myosin let go of actin; the power stroke follows Pi release. Rigor is the state with no ATP.
- Smooth muscle has actin and myosin but no troponin and no sarcomeres; Ca^{2+} works through calmodulin.
- Cardiac muscle is striated but involuntary; do not pair "striated" with "voluntary".

CHECK YOURSELF

– REMEMBER **Name the protein that Ca^{2+} binds in skeletal muscle and in smooth muscle.** 2 marks

Troponin C in skeletal (and cardiac) muscle; calmodulin in smooth muscle.

– UNDERSTAND **Why are skeletal muscle fibres multinucleate?** 2 marks

Each fibre forms by the fusion of many myoblasts during development, and their nuclei are kept.

– UNDERSTAND **Explain rigor mortis using the cross-bridge cycle.** 3 marks

After death ATP runs out. Myosin heads that have done their power stroke cannot detach without ATP, so they stay bound to actin and the muscle stays stiff until proteins break down.

– APPLY **With the filament lengths of the worked example, what is the I band at a sarcomere length of 3.0 µm?** 2 marks

I band = 3.0 − 1.6 = **1.4 µm**.

– ANALYZE **Why must heart cells be electrically coupled while skeletal fibres are not?** 4 marks

The heart must pump as one unit, so the spike from the pacemaker spreads cell to cell through gap junctions in the intercalated discs. Skeletal muscle needs graded force, so each fibre is driven by its own motor neuron and the brain chooses how many motor units to fire.

BEYOND THE SYLLABUS

Not for the exam. Duchenne muscular dystrophy comes from loss of dystrophin, a protein linking the actin cytoskeleton to the membrane, and gene therapies for it are now in clinical use. Cultured meat grows skeletal muscle cells on scaffolds in bioreactors, a growing biotech industry. Search next: "dystrophin gene therapy" and "cultivated meat bioreactor".

6.3 Cells of vision and pancreatic cells

Two more specialised cells show how the same signalling parts from Unit 5 are rewired for very different jobs. A rod or cone in the retina turns a photon into a change in membrane voltage. A pancreatic cell either secretes digestive enzymes into the gut or senses blood glucose and releases a hormone.

You need: G-protein coupled receptors and second messengers (Unit 5), the secretory pathway through ER and Golgi (Unit 3), and membrane potential (Chapter 6.1).

Rods and cones

Photoreceptors are neurons with a light-catching **outer segment**, a stack of membrane discs full of visual pigment, joined by a thin cilium to an **inner segment** rich in mitochondria, then a nucleus and a synaptic terminal. **Rods** (about 120 million per human eye) are very sensitive and work in dim light, but give no colour. **Cones** (about 6 million, packed in the fovea) need brighter light and give sharp, colour vision. Humans have three cone types, most sensitive to short (blue, about 420 nm), medium (green, about 530 nm) and long (red-yellow, about 560 nm) wavelengths.

Phototransduction: light closes channels

The rod pigment rhodopsin is a GPCR: the protein opsin with 11-cis-retinal (from vitamin A) bound inside. In the dark, cGMP holds cation channels open, so Na^+ and Ca^{2+} flow in (the "dark current"), the rod sits at about −40 mV and releases glutamate steadily.

1. A photon isomerises 11-cis-retinal to all-trans-retinal; rhodopsin changes shape (activated rhodopsin).
2. Activated rhodopsin activates many molecules of the G protein transducin.
3. Transducin's α subunit switches on phosphodiesterase (PDE6), which hydrolyses cGMP.

4. cGMP falls, the cGMP-gated channels close, and the rod hyperpolarises towards about −70 mV.
5. Glutamate release drops; the next neurons (bipolar cells) read this drop as "light".

Each step multiplies the signal, which is why a rod can respond to a single photon. Notice the twist: light makes a photoreceptor *hyperpolarise*, the opposite of most sensory cells.

Pancreatic cells: exocrine and endocrine

Nearly all of the pancreas is **exocrine** (the islets are only about 1–2% of its mass): acinar cells that make digestive enzymes (trypsinogen, amylase, lipase) on rough ER, pack them in the Golgi into zymogen granules at the cell apex, and release them by exocytosis into ducts. George Palade worked out the secretory pathway by following labelled protein through these very cells. Enzymes leave as inactive precursors so they do not digest the pancreas itself.

Scattered among them are the **islets of Langerhans**, the endocrine part: **β cells** make insulin, **α cells** glucagon, **δ cells** somatostatin and **PP cells** pancreatic polypeptide. β cells are the most numerous islet cell.

A β cell is a glucose sensor wired to a secretory machine:

1. Glucose enters through glucose transporters and is phosphorylated by glucokinase, which works in proportion to blood glucose.
2. Glycolysis and mitochondria raise the ATP/ADP ratio.
3. ATP closes ATP-sensitive K^+ channels (K_{ATP}); less K^+ leaves, so the cell depolarises.
4. Voltage-gated Ca^{2+} channels open; Ca^{2+} triggers exocytosis of insulin granules.

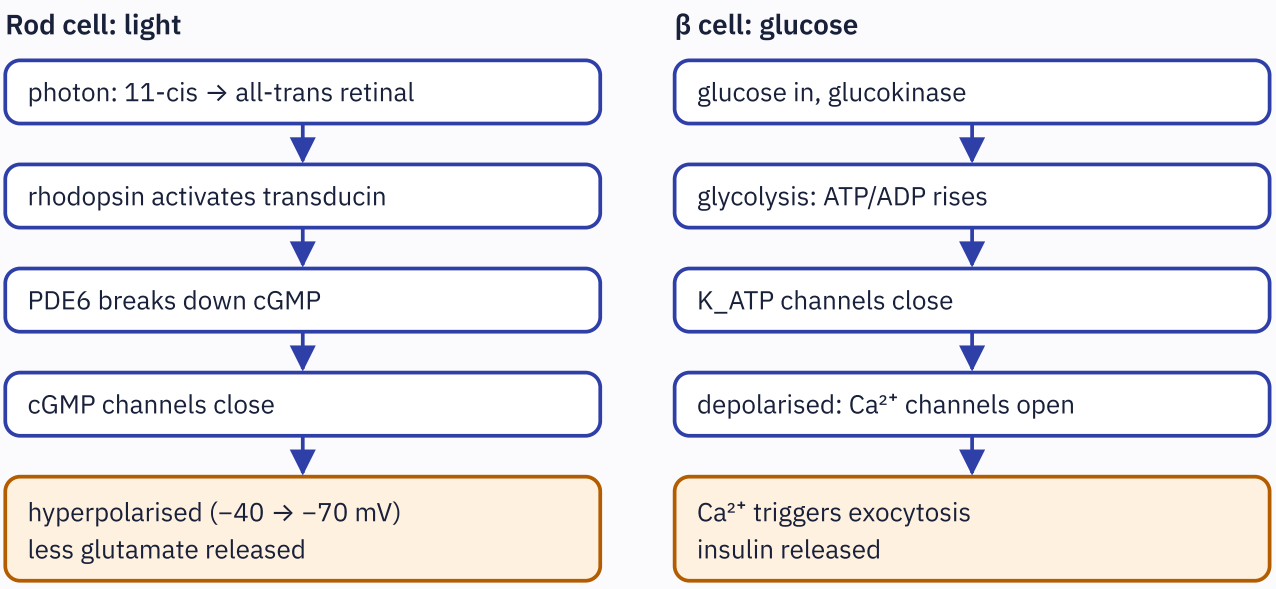


Figure 6.3.1 The two cascades side by side. In both, a chemical change closes a channel; in the rod the cell hyperpolarises and releases less transmitter, in the β cell it depolarises and releases more hormone.

WORKED EXAMPLE

Question (6 marks). (a) Sulfonylurea drugs, used in type 2 diabetes, bind and close the K_{ATP} channel. Predict their effect on insulin release. (b) Some babies are born with a K_{ATP} channel that stays open even when ATP is high. Predict the effect, and suggest a treatment.

1. (a) Closing K_{ATP} mimics high ATP: K^+ outflow drops, the β cell depolarises, Ca^{2+} channels open and insulin is released, even at modest glucose. So sulfonylureas **raise insulin secretion**; their risk is low blood sugar.
2. (b) If the channel cannot close, glucose metabolism raises ATP but the cell stays hyperpolarised. Ca^{2+} channels stay shut and **little insulin is released**: the baby has diabetes from birth (neonatal diabetes).
3. Treatment logic: a drug that closes the channel by another route can restore depolarisation. Many such patients are indeed treated with high-dose sulfonylureas instead of insulin injections.

EXAM TRAPS

- Light hyperpolarises rods and cones; it does not depolarise them. Glutamate release falls in light.
- cGMP, not cAMP, is the second messenger in photoreceptors; PDE breaks it down.
- β cells make insulin and α cells make glucagon; the letters are swapped often.
- Rods: dim light, no colour, one pigment. Cones: bright light, colour, three pigments.

CHECK YOURSELF

– REMEMBER **Name the four cell types of an islet and the hormone each makes.** 2 marks

β: insulin; α: glucagon; δ: somatostatin; PP: pancreatic polypeptide.

– REMEMBER **What are the two parts of rhodopsin?** 1 mark

The protein opsin and the chromophore 11-cis-retinal.

– UNDERSTAND **Why does vitamin A deficiency cause night blindness?** 3 marks

Retinal is made from vitamin A. Without it rods cannot rebuild rhodopsin, and rods are the cells that work in dim light, so vision fails first at night.

– APPLY **A drug blocks PDE6 in rods. What happens to the dark current when light falls on the rod?** 3 marks

cGMP cannot be broken down, so the cGMP-gated channels stay open; the dark current continues, the rod does not hyperpolarise and the light is not signalled.

– ANALYZE **Compare how an acinar cell and a β cell decide when to secrete.** 5 marks

Both store product in granules made through ER and Golgi and release by Ca^{2+}-triggered exocytosis. The acinar cell responds to hormones and nerves from the gut after a meal; the β cell reads blood glucose itself through its metabolism and the K_{ATP} channel. Acinar products go into ducts; insulin goes into blood.

BEYOND THE SYLLABUS

Not for the exam. Researchers now make insulin-secreting β-like cells from stem cells (see Unit 4, Stem cells) and transplant them, and some type 1 diabetes trials have reported patients producing their own insulin again. Gene therapy for an inherited retinal disease (RPE65 mutation) is an approved product. Search next: "stem cell derived beta cells" and "retinal gene therapy RPE65".

6.4 Light and fluorescence microscopy

Most cells are 10 to 100 µm across, too small for the eye (which resolves about 0.1 mm), so cell biology began with the light microscope. A light microscope uses glass lenses to magnify an image formed by visible light; a fluorescence microscope lights the sample with one colour and collects the different colour that tagged molecules glow with, so you see only what you labelled.

You need: cell sizes and organelles (Unit 1, Overview of cell organelles and compartments).

Magnification is not resolution

In a compound microscope the **objective** lens near the sample forms a magnified image, and the **eyepiece** (ocular) magnifies it again. Total magnification = objective × eyepiece, so a 40× objective with a 10× eyepiece gives 400×. Below the stage, the **condenser** focuses light from the lamp onto the sample, and its diaphragm controls the cone of light.

Magnification only makes the picture bigger. **Resolution** is the smallest distance d at which two points still look like two. Because light is a wave, a point spreads into a small blur, and two blurs closer than about d merge. The Rayleigh criterion gives:

$$d = 0.61\,\lambda / NA \qquad NA = n \cdot \sin\theta$$

Here λ is the wavelength of light, NA the numerical aperture of the objective (engraved on its barrel), n the refractive index of the medium between lens and sample, and θ half the angle of the cone of light the objective accepts. A smaller d is better. Two ways to improve it: use shorter λ (blue rather than red) or larger NA. Air has n = 1.0, so a dry objective's NA stays below 1. Immersion oil has n ≈ 1.515, close to glass, so oil objectives reach NA ≈ 1.4. This sets the classic limit of about 0.2 µm: mitochondria and bacteria can be seen; ribosomes (about 25 nm)

and membranes cannot. Magnifying beyond about 1000 × NA adds size but no new detail ("empty magnification").

Getting contrast

Living cells are mostly water and nearly transparent, so in plain **bright-field** they show little detail. Four answers:

- **Staining** fixed cells with dyes: methylene blue or haematoxylin colour nuclei; eosin colours cytoplasm pink; Gram stain separates bacteria. Staining usually kills the cell.
- **Phase contrast** (Zernike, 1930s): converts the tiny delay that light suffers passing through denser parts (nucleus, organelles) into differences in brightness. Ideal for live, unstained cells in culture.
- **Differential interference contrast (DIC**, Nomarski): uses polarised light to show gradients of optical thickness, giving a shaded, 3D-looking image.
- **Dark-field**: blocks direct light, so only light scattered by the sample enters the objective; objects shine bright on black.

Sample preparation, as in a teaching lab: a **wet mount** is a drop of live sample under a coverslip, for motility and shape; a **dry, fixed smear** is spread thin, air-dried, heat- or chemically fixed, then stained. Thick tissues are fixed, embedded in paraffin wax and cut into sections a few micrometres thick with a microtome before staining.

Fluorescence microscopy

A **fluorophore** absorbs a photon of one wavelength (excitation) and, a few nanoseconds later, emits a photon of longer wavelength (emission), because some energy is lost as heat in between. This shift to longer wavelength is the **Stokes shift**, and it is the whole trick: if you block the excitation colour and pass only the emission colour, only labelled structures appear, glowing on a black background.

Most instruments are **epifluorescence** microscopes: light comes down through the objective and the glow goes back up through the same objective. The **filter cube** has three parts:

1. **Excitation filter**: passes only the band that excites the dye (blue for green-emitting dyes).
2. **Dichroic mirror** at 45°: reflects the short excitation wavelengths down to the sample but lets the longer emission wavelengths pass up.
3. **Emission (barrier) filter**: removes any leftover excitation light, passing only the emission band to the eye or camera.

Common labels: **DAPI** and Hoechst bind DNA and glow blue (excited by UV light near 360 nm); **FITC** and Alexa Fluor 488 glow green (excited near 490 nm); rhodamine-type dyes glow red.

Green fluorescent protein (GFP), from the jellyfish *Aequorea victoria*, can be fused to any protein by genetic engineering, so a living cell shows where that protein is (Chapter 6.8). Shimomura, Chalfie and Tsien shared the 2008 Nobel Prize in Chemistry for it. Two practical limits: **photobleaching** (the fluorophore is destroyed by strong light, so the signal fades) and out-of-focus glow from thick samples, which the confocal microscope removes (Chapter 6.5).

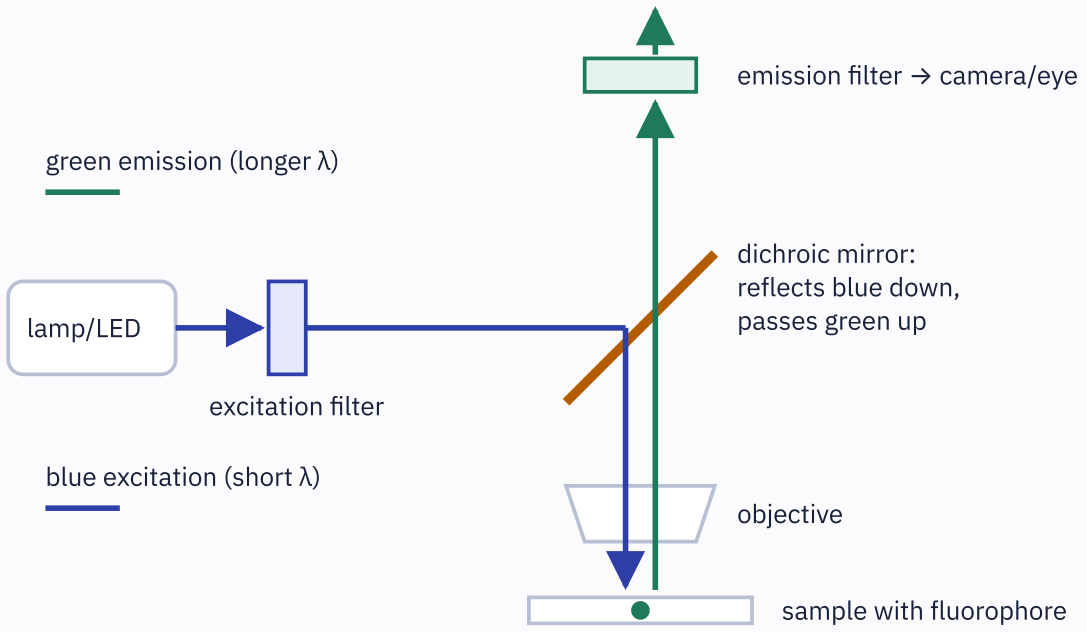


Figure 6.4.1 The filter cube of an epifluorescence microscope. Blue light (short wavelength) is reflected down by the dichroic mirror onto the sample; the green glow (longer wavelength, the Stokes shift) passes straight up through the mirror and the emission filter. Notice that one objective both lights and views the sample.

WORKED EXAMPLE 1: RESOLUTION

Question (4 marks). Find the resolution with green light (λ = 550 nm) for (a) a dry 40× objective, NA 0.65, and (b) an oil 100× objective, NA 1.40. Can either separate two vesicles 300 nm apart?

1. (a) d = 0.61 × 550 / 0.65 = 335.5 / 0.65 = **516 nm ≈ 0.52 µm**.
2. (b) d = 0.61 × 550 / 1.40 = 335.5 / 1.40 = **240 nm ≈ 0.24 µm**.
3. 300 nm is larger than 240 nm but smaller than 516 nm, so only the **oil objective** shows two vesicles; the dry lens shows one blur.

WORKED EXAMPLE 2: COUNTING CELLS WITH A HAEMOCYTOMETER

Question (4 marks). A yeast culture is diluted 1:10 and loaded on an improved Neubauer haemocytometer. Counts in the four large corner squares (each 1 mm × 1 mm, chamber depth 0.1 mm) are 52, 48, 55 and 45. Find the cells per mL in the original culture.

1. Mean count per large square = (52 + 48 + 55 + 45) / 4 = 200 / 4 = 50 cells.
2. Volume over one large square = 1 mm × 1 mm × 0.1 mm = 0.1 mm^3 = 0.1 µL = 1×10^{-4} mL.
3. Cells per mL in the diluted sample = 50 / 10^{-4} = 5.0×10^5.
4. Undo the dilution: $5.0 \times 10^5 \times 10$ = **5.0×10^6 cells/mL**.

EXAM TRAPS

- Higher magnification does not mean better resolution; resolution depends on λ and NA only.
- Smaller d means better resolving power. Students often say "resolution increased" when d grew.
- Emission is at a longer wavelength than excitation, never shorter.
- The dichroic mirror reflects excitation and transmits emission; it is not an ordinary mirror or a filter that blocks both.
- In the haemocytometer, one large square holds 10^{-4} mL; forgetting the depth (0.1 mm) gives an answer 10 times too small.

CHECK YOURSELF

– REMEMBER **Write the Rayleigh equation and define NA.** 2 marks

d = 0.61 λ / NA, with NA = n sin θ (n: refractive index of the medium; θ: half-angle of the light cone the objective accepts).

– REMEMBER **Name the three parts of a fluorescence filter cube.** 3 marks

Excitation filter, dichroic mirror, emission (barrier) filter.

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

Oil (n ≈ 1.515) matches glass, so light bent at wide angles is not lost by refraction at an air gap; the objective collects a wider cone, NA rises to about 1.4 and d falls.

– UNDERSTAND **Which method would you use to watch unstained live cells divide, and why?** 2 marks

Phase contrast (or DIC): it turns differences in optical path into brightness, so transparent living cells show detail without stains that would kill them.

– APPLY **Find d for blue light (450 nm) with NA 1.40.** 2 marks

d = 0.61 × 450 / 1.40 = **196 nm ≈ 0.20 µm**; shorter λ gives finer resolution.

– APPLY **Haemocytometer counts of 30, 34, 28 and 28 cells in the four large squares, undiluted sample. Cells per mL?** 3 marks

Mean = 120 / 4 = 30; 30 / 10^{-4} = **3.0×10^5 cells/mL**.

– ANALYZE **A student sees a dim green signal everywhere in a thick tissue slice and it fades over a minute. Explain both observations and suggest fixes.** 5 marks

Everywhere-glow: fluorescence from planes above and below focus is also collected in a widefield microscope; use thinner sections or a confocal microscope. Fading: photobleaching; reduce light intensity or exposure, use an antifade mounting medium or a more stable dye.

BEYOND THE SYLLABUS

Not for the exam. Super-resolution methods such as STED, PALM and STORM beat the 0.2 µm limit by switching fluorophores on and off so that nearby molecules glow at different times; they won the 2014 Nobel Prize in Chemistry. Light-sheet microscopy lights only a thin plane, so whole embryos can be filmed for days with little bleaching. Expansion microscopy swells the sample itself in a gel. Search next: "STED microscopy", "light-sheet microscopy" and "expansion microscopy".

6.5 Electron and confocal microscopy

When light cannot resolve a structure, use electrons; when a fluorescent sample is too thick and blurred, use a pinhole. Electron microscopes (TEM and SEM) replace light with an electron beam whose wavelength is about 100,000 times shorter, and show membranes, ribosomes and viruses. The confocal microscope keeps fluorescence but rejects out-of-focus light, giving sharp optical slices and 3D images of cells.

You need: resolution and fluorescence (Chapter 6.4).

Transmission electron microscope (TEM)

A heated filament or field-emission gun produces electrons, which are accelerated by a high voltage (typically 80 to 300 kV) down a column kept under **vacuum** (air would scatter them). Electromagnetic coils act as lenses. Electrons pass *through* a very thin sample; dense regions scatter electrons away and look dark, giving a 2D projection on a fluorescent screen or camera. For biological sections the practical resolution is a few nanometres, set by the sample, not the beam.

Sample preparation for thin-section TEM:

1. **Fixation** with glutaraldehyde (cross-links proteins), then osmium tetroxide (fixes and stains lipids).
2. **Dehydration** through rising concentrations of ethanol or acetone, since water boils in vacuum.
3. **Embedding** in epoxy resin, which hardens into a block.
4. **Ultrathin sectioning** (about 50–100 nm) on an ultramicrotome with a diamond or glass knife.
5. **Heavy-metal staining** with uranyl acetate and lead citrate, which bind membranes and nucleic acids and scatter electrons.

Other routes: **negative staining** surrounds viruses or protein complexes with heavy-metal salt so they appear light on dark; **cryo-EM** freezes samples so fast that water forms glass-like (vitreous) ice and averages thousands of images to solve protein structures (2017 Nobel Prize in Chemistry).

Scanning electron microscope (SEM)

An SEM scans a focused beam across the *surface* of the sample. Secondary electrons knocked out of the surface are collected and turned into an image with a striking 3D look, ideal for cell shape, microvilli, cilia and bacteria on a surface. Samples are fixed, dehydrated, dried by **critical-point drying** (to avoid collapse from surface tension) and coated with a thin layer of gold or another metal by sputter coating so they conduct. Resolution is usually lower than TEM, a few nanometres or more.

Confocal microscope

In a widefield fluorescence microscope the whole depth of the sample glows, so a thick specimen gives a blur. A confocal microscope (the idea patented by Marvin Minsky) focuses a **laser** to a point in the sample and places a **pinhole** in front of the detector at the plane conjugate to that focal point. Light from the focal point passes the pinhole; light from planes above and below is out of focus at the pinhole and is blocked. Scanning the point across the field builds one sharp **optical section**; moving the focus in steps gives a **z-stack** that software turns into a 3D image. The price: slower imaging and more photobleaching than widefield.

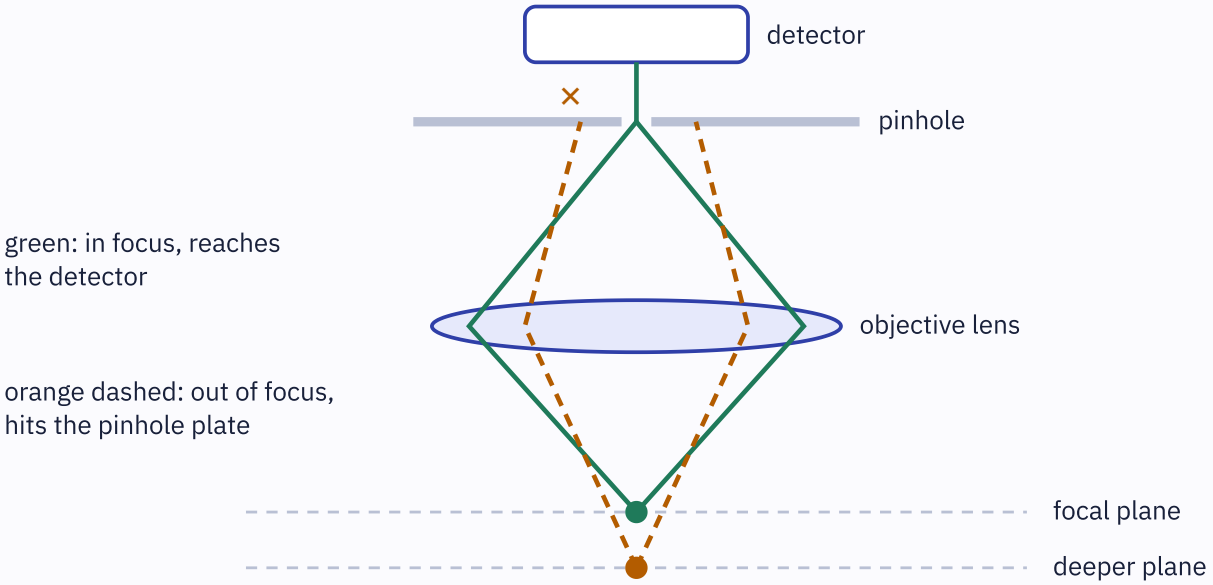


Figure 6.5.1 Why a confocal image is sharp (laser path omitted). Light from the focal plane (green) converges exactly on the pinhole and reaches the detector; light from a deeper plane (orange) converges in the wrong place and is mostly stopped by the plate.

WORKED EXAMPLE

Question (5 marks). The de Broglie wavelength of an electron accelerated through V volts is about $\lambda = 1.226 / \sqrt{V}$ nm (ignoring relativity). Find λ at 100 kV, compare it with green light (550 nm), and explain why biological TEM images do not reach this limit.

1. $\sqrt{V} = \sqrt{100{,}000} = 316.2$.
2. $\lambda = 1.226 / 316.2 =$ **0.0039 nm** (the relativistic value is a little shorter, about 0.0037 nm).
3. Ratio: $550 / 0.0039 \approx 1.4 \times 10^5$, so the electron wavelength is over 100,000 times shorter.
4. In practice, electron lenses have a small aperture and large aberrations, and biological samples are damaged by the beam, have low contrast and are limited by fixation, sectioning and stain grain. So stained sections resolve a few nanometres, still about 100 times better than the light microscope (about 200 nm).

EXAM TRAPS

- TEM: electrons pass through a thin section, 2D internal detail. SEM: surface scanning, 3D-looking surface. Do not swap them.
- Living cells cannot be seen in a conventional EM: vacuum, fixation and dehydration are required.
- The confocal pinhole sits in front of the detector, not in front of the lamp alone; it is the detector pinhole that rejects out-of-focus light.
- Electron lenses are electromagnetic coils, not glass.

CHECK YOURSELF

– REMEMBER **Name the two heavy-metal stains used for TEM sections.** 2 marks

Uranyl acetate and lead citrate (osmium tetroxide also fixes and stains lipids).

– UNDERSTAND **Why must an electron microscope column be under vacuum?** 2 marks

Gas molecules would scatter the electrons and blur or stop the beam (and the filament would oxidise).

– UNDERSTAND **Why are SEM samples coated with gold?** 2 marks

The metal makes the surface conductive, so charge from the beam drains away, and it gives a strong secondary-electron signal.

– APPLY **Choose a method: (a) shape of pollen grain surfaces; (b) cristae inside mitochondria; (c) 3D shape of a GFP-tagged Golgi in a living cell.** 3 marks

(a) SEM; (b) TEM of thin sections; (c) confocal microscopy with a z-stack.

– ANALYZE **What happens to a confocal image if the pinhole is opened very wide, and why?** 3 marks

More light reaches the detector (brighter), but out-of-focus light also passes, so the optical section gets thicker and the image blurs towards a widefield picture.

BEYOND THE SYLLABUS

Not for the exam. Cryo-electron tomography images frozen cells from many angles to see molecular machines in their native place. Volume EM (serial block-face SEM) maps the wiring of whole brain regions. Two-photon microscopes excite with infrared pulses to image deep inside living tissue. Search next: "cryo-electron tomography" and "two-photon microscopy".

6.6 Cell fractionation and isolation of organelles

Cell fractionation breaks cells open gently and separates their parts, so that nuclei, mitochondria or ER membranes can be studied in a test tube. It is how biologists proved what each organelle does: Albert Claude, Christian de Duve and George Palade shared the 1974 Nobel Prize in Physiology or Medicine for this work, which found lysosomes and peroxisomes. The method has two steps, breaking the cells (homogenisation) and sorting the pieces by centrifugation.

You need: organelle sizes and functions (Unit 3: Mitochondria; Endoplasmic reticulum; Lysosomes, peroxisomes and vacuoles).

Step 1: homogenisation

The aim is to break the plasma membrane (and the cell wall in plants or fungi) but keep organelles whole. Common methods:

- **Potter–Elvehjem or Dounce homogeniser**: a pestle turning or moving in a close-fitting glass tube; shear forces tear soft tissue such as liver.

- **Blender** for tough tissue; **sonication** (ultrasound) and the **French press** (forcing cells through a narrow valve under high pressure) for bacteria and yeast.
- **Osmotic lysis**: cells without a wall swell and burst in a hypotonic solution.
- **Enzymes**: cellulase and pectinase for plant walls, lysozyme for bacteria.

The homogenisation buffer matters as much as the force. It is **isotonic** (commonly 0.25 M sucrose; mannitol is also used), so organelles neither swell nor shrink; **buffered** near pH 7.4; **ice-cold** (0–4 °C) to slow enzymes; and often contains protease inhibitors and EDTA (which binds metal ions that activate degrading enzymes).

Step 2: differential centrifugation

In a centrifuge, a particle feels a force many times gravity. This **relative centrifugal force (RCF)**, quoted as "× g", depends on the radius r and the speed:

$$\text{RCF} = 1.118 \times 10^{-5} \times r \times (\text{rpm})^2 \qquad (r \text{ in cm})$$

Larger and denser particles sediment faster. A particle's sedimentation rate per unit field is its **sedimentation coefficient**, $s = v / (\omega^2 r)$, where v is its velocity, ω the angular speed in rad/s and r the distance from the axis; s is measured in svedbergs ($1\ \text{S} = 10^{-13}$ s). That is why ribosomes are called 70S and 80S.

Differential centrifugation spins the homogenate at a series of rising speeds. After each spin the pellet is kept and the supernatant moves to the next, faster spin. Typical values (protocols differ):

1. About 1,000 × g for 10 min: nuclei, unbroken cells and large debris pellet.
2. About 10,000–20,000 × g for 20 min: mitochondria, lysosomes and peroxisomes (and chloroplasts from plant tissue).
3. About 80,000–100,000 × g for 1 h (ultracentrifuge): **microsomes**, small vesicles formed from ER and plasma membrane during homogenisation.
4. About 150,000 × g for several hours: ribosomes and large macromolecular complexes. The final supernatant is the **cytosol**.

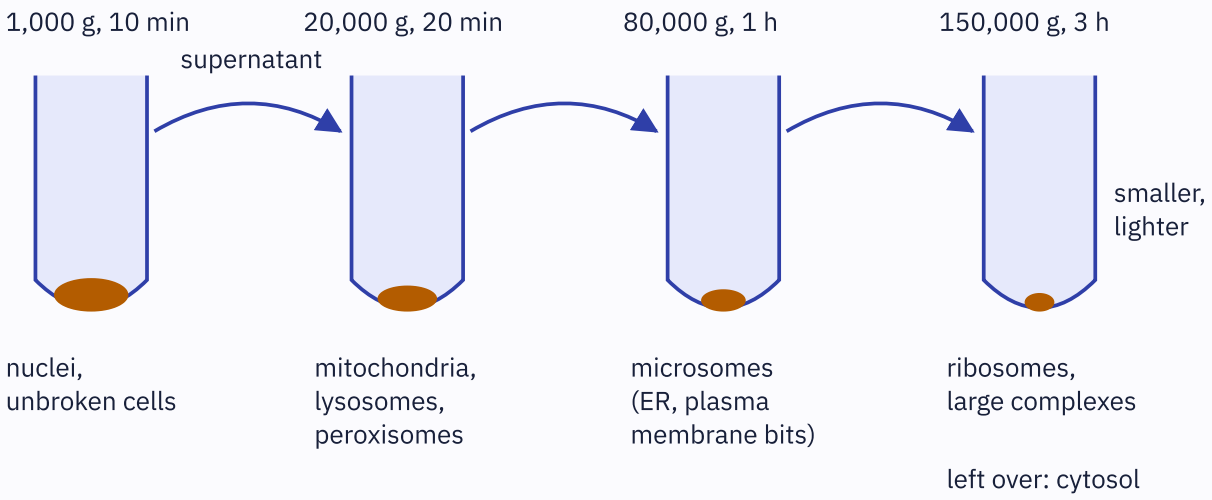


Figure 6.6.1 Differential centrifugation of a liver homogenate. Each spin is faster and longer than the one before; the pellet (orange) is kept and the supernatant moves right. Notice that pellets get smaller as the particles do, and that the 20,000 g pellet mixes three organelles of similar size.

Density-gradient centrifugation: cleaning up a fraction

Differential pellets are enriched, not pure: mitochondria, lysosomes and peroxisomes overlap in size. A **density gradient** separates them further.

- **Rate-zonal** (velocity) centrifugation: the sample is layered on a shallow gradient (for example 5–20% sucrose) and spun for a set time. Particles move as bands by size and shape (their s value); the run is stopped before they reach the bottom. Used for ribosomal subunits and polysomes.
- **Isopycnic** (equilibrium) centrifugation: a steep gradient (sucrose, Percoll or Nycodenz for organelles; caesium chloride for DNA) is spun until each particle stops at the layer whose density equals its own. Separation is by density only, not size. Peroxisomes are denser than mitochondria and band lower.

Checking the fractions: marker enzymes

Each organelle has an enzyme or molecule found almost nowhere else. Measuring it in every fraction tells you where that organelle went and how pure each fraction is.

ORGANELLE	MARKER
Nucleus	DNA
Mitochondria	Succinate dehydrogenase, cytochrome c oxidase
Lysosomes	Acid phosphatase

ORGANELLE	MARKER
Peroxisomes	Catalase
Endoplasmic reticulum (microsomes)	Glucose-6-phosphatase
Golgi	Galactosyltransferase
Plasma membrane	Na^+/K^+-ATPase, 5′-nucleotidase
Cytosol	Lactate dehydrogenase

Purity is reported as **specific activity** (enzyme units per mg protein). **Enrichment** (fold purification) = specific activity in the fraction ÷ specific activity in the homogenate, and **yield** = total units in the fraction ÷ total units in the homogenate × 100%. Today, Western blots for organelle marker proteins (Chapter 6.8) are also used.

WORKED EXAMPLE 1: SETTING THE CENTRIFUGE

Question (4 marks). A rotor has a radius of 8.0 cm. What speed in rpm gives (a) 10,000 × g and (b) 100,000 × g?

1. Rearrange: rpm = √[RCF / (1.118 × 10^{-5} × r)].
2. 1.118 × 10^{-5} × 8.0 = 8.944 × 10^{-5}.
3. (a) rpm = √(10,000 / 8.944 × 10^{-5}) = √(1.118 × 10^{8}) ≈ **10,600 rpm**.
4. (b) rpm = √(100,000 / 8.944 × 10^{-5}) = √(1.118 × 10^{9}) ≈ **33,400 rpm**. Ten times the force needs only √10 ≈ 3.2 times the speed.

WORKED EXAMPLE 2: ENRICHMENT AND YIELD

Question (5 marks). A liver homogenate has 500 mg protein and 25 units of succinate dehydrogenase (SDH). The 20,000 × g pellet has 20 mg protein and 12 units of SDH. Find the specific activities, the enrichment and the yield of mitochondria. The pellet also shows high catalase activity: what does that mean?

1. Homogenate specific activity = 25 / 500 = 0.05 U/mg.
2. Pellet specific activity = 12 / 20 = 0.60 U/mg.
3. Enrichment = 0.60 / 0.05 = **12-fold**.
4. Yield = 12 / 25 × 100 = **48%**; the rest of the mitochondria were lost in other fractions or broken.
5. Catalase marks peroxisomes, which co-sediment with mitochondria. The pellet is contaminated; an isopycnic gradient would separate them.

EXAM TRAPS

- Quote centrifuge settings in × g, not rpm: the same rpm gives different forces in rotors of different radius.
- Microsomes are not an organelle in the living cell; they are vesicles made by breaking the ER and other membranes.
- Rate-zonal separates by size (stopped before equilibrium); isopycnic separates by density (run to equilibrium). This is the classic 8-mark comparison.
- In differential centrifugation the pellet is kept and the supernatant spun again; students reverse this.
- High enrichment with low yield is common and is not an error.

CHECK YOURSELF

– REMEMBER **Give the marker enzyme for lysosomes, peroxisomes and ER.** 3 marks

Acid phosphatase; catalase; glucose-6-phosphatase.

– REMEMBER **What is one svedberg?** 1 mark

10^{-13} seconds, the unit of sedimentation coefficient.

– UNDERSTAND **Why is the homogenisation buffer isotonic and ice-cold?** 3 marks

Isotonic sucrose stops organelles swelling and bursting by osmosis; the cold slows proteases, lipases and nucleases released from broken lysosomes and cytosol.

– UNDERSTAND **Why do mitochondria and peroxisomes end up in the same differential pellet, and how are they separated?** 3 marks

They are similar in size, so they sediment at similar speeds. They differ in density, so isopycnic gradient centrifugation bands them at different heights.

– APPLY **A rotor of radius 10 cm runs at 15,000 rpm. What is the RCF?** 2 marks

RCF = $1.118 \times 10^{-5} \times 10 \times (15{,}000)^2 = 1.118 \times 10^{-4} \times 2.25 \times 10^8 \approx$ **25,200 × g**.

– APPLY **A homogenate has catalase specific activity 0.2 U/mg; a gradient fraction has 3.0 U/mg. What is the enrichment?** 2 marks

3.0 / 0.2 = **15-fold**.

– ANALYZE **After homogenising liver too vigorously, acid phosphatase appears mostly in the final supernatant. Explain and suggest a fix.** 4 marks

Harsh homogenisation broke lysosome membranes, so the soluble enzyme leaked into the cytosol and stays in the supernatant instead of pelleting with lysosomes. Use fewer or gentler pestle strokes, keep the buffer isotonic and cold, and check intactness with a latency assay.

BEYOND THE SYLLABUS

Not for the exam. Organelle proteomics combines gradient fractionation with mass spectrometry: by matching each protein's profile across fractions to known markers, thousands of proteins can be assigned to organelles in one experiment ("spatial proteomics"). Immuno-isolation pulls out one organelle with antibody-coated magnetic beads for very pure samples. Search next: "spatial proteomics LOPIT" and "organelle immunoprecipitation".

6.7 Flow cytometry

A flow cytometer pushes cells one at a time through a laser beam and measures, for each cell, how it scatters light and how brightly it glows in several colours. It reads thousands of cells per second, so instead of looking at a few cells under a microscope you get numbers

for a whole population: what percentage are CD4 T cells, how many are dividing, how many are dying. A cell sorter (FACS) can then separate the cells you want.

You need: fluorescence and fluorophores (Chapter 6.4); apoptosis and the cell cycle (Unit 4).

The three systems

1. **Fluidics.** The cell suspension is injected into a faster stream of sheath fluid. **Hydrodynamic focusing** squeezes the sample into a narrow core, so cells pass the laser in single file.
2. **Optics.** One or more lasers (488 nm blue is the classic) hit each cell. Light scattered at small angles along the beam is **forward scatter (FSC)**, which rises with cell size. Light scattered at about 90° is **side scatter (SSC)**, which rises with internal complexity or granularity. Fluorescence from dyes or labelled antibodies is split by dichroic mirrors and filters into colour channels.
3. **Electronics.** Photodetectors (photomultiplier tubes or photodiodes) turn each flash into a voltage pulse. The pulse height, area and width are digitised for every channel of every cell (each cell is an "event").

Reading the data: gates and plots

Data are shown as **histograms** (one parameter: number of cells against intensity) or **dot plots** (two parameters, one dot per cell). A **gate** is a region you draw to keep only some events: first FSC against SSC to keep intact cells and drop debris, then a pulse area against width plot to drop doublets (two cells stuck together), then the fluorescence of interest. Because dye emission spectra overlap, part of one dye's signal leaks into the next channel; **compensation** subtracts that leak using single-stained controls. Blood is the classic example: on FSC against SSC, lymphocytes (small, smooth), monocytes (larger) and granulocytes (high SSC from granules) form three separate clouds.

Common uses

- **Immunophenotyping**: fluorescent antibodies against surface CD markers count cell types; CD4 T-cell counts guide HIV care, and marker panels classify leukaemias.
- **Cell cycle**: a DNA dye such as propidium iodide (PI) on fixed cells gives a histogram with a G1 peak (2N DNA), a G2/M peak at twice the intensity (4N) and S phase in between.
- **Apoptosis**: annexin V binds phosphatidylserine, which flips to the outer leaflet early in apoptosis; PI enters only cells whose membrane has broken.
- **Sorting (FACS)**: the stream is vibrated into droplets; a droplet holding a wanted cell is given a charge and deflected by charged plates into a collection tube.

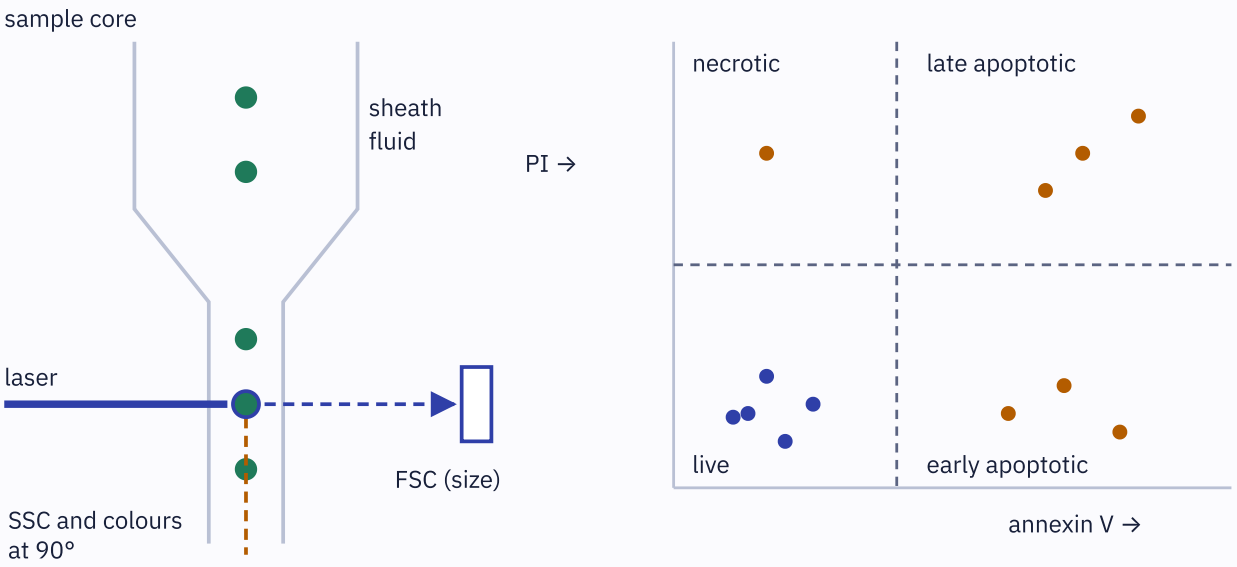


Figure 6.7.1 Left: hydrodynamic focusing lines cells up in single file; each cell crossing the laser sends scattered light forward (FSC) and sideways (SSC, plus fluorescence). Right: an annexin V against PI dot plot; each dot is one cell, and the quadrant it falls in tells you its state.

WORKED EXAMPLE

Question (5 marks). Cancer cells are treated with a drug and stained with annexin V–FITC and PI. Of 20,000 gated events: 14,000 are annexin$^-$ PI$^-$, 3,000 annexin$^+$ PI$^-$, 2,400 annexin$^+$ PI$^+$ and 600 annexin$^-$ PI$^+$. Give the percentage in each state and the total apoptotic fraction.

1. Live (annexin$^-$ PI$^-$): 14,000 / 20,000 = **70%**.
2. Early apoptotic (annexin$^+$ PI$^-$: PS exposed, membrane intact): 3,000 / 20,000 = **15%**.
3. Late apoptotic or secondary necrotic (annexin$^+$ PI$^+$): 2,400 / 20,000 = **12%**.
4. Necrotic or damaged (annexin$^-$ PI$^+$): 600 / 20,000 = **3%**.
5. Total apoptotic = 15 + 12 = **27%**. Compare with an untreated control before saying the drug caused it.

EXAM TRAPS

- FSC relates to size, SSC to granularity or internal complexity; not the other way round.
- Flow cytometry gives no image of the cell (imaging flow cytometers aside); it gives numbers per cell.
- FACS means fluorescence-activated cell sorting; analysis alone is flow cytometry.
- Annexin V binds phosphatidylserine on the outside of the cell; PI marks broken membranes, not apoptosis itself.

CHECK YOURSELF

– REMEMBER **What do FSC and SSC measure?** 2 marks

FSC: roughly cell size. SSC: internal complexity or granularity.

– UNDERSTAND **What is hydrodynamic focusing and why is it needed?** 3 marks

The sample stream is injected into faster sheath fluid, which narrows it to a thin core so cells pass the laser one at a time; otherwise two cells would be read as one event.

– UNDERSTAND **Why is compensation needed in multicolour experiments?** 2 marks

Emission spectra overlap, so part of one dye's light is detected in another dye's channel; compensation subtracts that spillover.

– APPLY **In a PI cell-cycle histogram the G1 peak is at intensity 200. Where is the G2/M peak, and where are S-phase cells?** 2 marks

G2/M at about **400** (twice the DNA); S-phase cells lie between 200 and 400.

– ANALYZE **Why do you gate on FSC against SSC before looking at fluorescence?** 3 marks

Debris, dead fragments and clumps also give fluorescence events. Gating on intact single cells by size and complexity first removes them, so percentages refer to real cells.

BEYOND THE SYLLABUS

Not for the exam. Spectral flow cytometers record each cell's whole emission spectrum and unmix 30 or more colours. Mass cytometry (CyTOF) labels antibodies with metal isotopes and reads them by mass spectrometry. In CAR-T cell therapy, flow cytometry checks every manufactured batch. Search next: "spectral flow cytometry" and "mass cytometry CyTOF".

6.8 Immunostaining and protein localization

Immunostaining uses antibodies to find one protein among thousands in a fixed cell or tissue and make it visible, by a fluorescent dye (immunofluorescence) or a coloured enzyme product (immunohistochemistry). It answers the question "where in the cell is my protein?": in the nucleus, on mitochondria, at the plasma membrane. Protein localisation also uses GFP fusions in live cells and fractionation with Western blots.

You need: fluorescence microscopy (Chapter 6.4), confocal microscopy (Chapter 6.5) and cell fractionation (Chapter 6.6).

Antibodies as probes

An IgG antibody is a Y-shaped protein of two heavy and two light chains. The two tips (the **Fab** arms) bind one small region of the target, the **epitope**; the stem (the **Fc** region) is the same for all antibodies of one species and class. A **monoclonal** antibody comes from one B-cell clone and binds one epitope; a **polyclonal** serum is a mixture binding several epitopes, which gives a stronger but less defined signal.

- **Direct** staining: the primary antibody itself carries the label. Fewer steps, weaker signal.
- **Indirect** staining: an unlabelled primary antibody (say, raised in rabbit) binds the target; a labelled **secondary** antibody (goat anti-rabbit IgG) binds the primary's Fc. Several secondaries bind each primary, so the signal is amplified, and one labelled secondary works with any rabbit primary.

The immunofluorescence protocol

1. **Fix**: 4% paraformaldehyde cross-links proteins and holds them in place; cold methanol instead precipitates proteins and also permeabilises.
2. **Permeabilise** (after aldehyde fixation): a detergent such as Triton X-100 or saponin opens membranes so antibodies (about 150 kDa) reach inside.
3. **Block** with BSA or serum from the secondary's host species, which coats sticky sites and cuts background.
4. **Primary antibody**, diluted (often 1:100 to 1:1000), usually 1 h at room temperature or overnight at 4 °C; wash.
5. **Secondary antibody** with a fluorophore, in the dark; wash.

6. **Counterstain** nuclei with DAPI, mount in antifade medium and image by fluorescence or confocal microscopy.

Controls make the result believable: a **secondary-only** control (no primary) shows background; an **isotype** control for monoclonals; and the best test of specificity, cells where the gene is knocked out or knocked down, which should lose the signal.

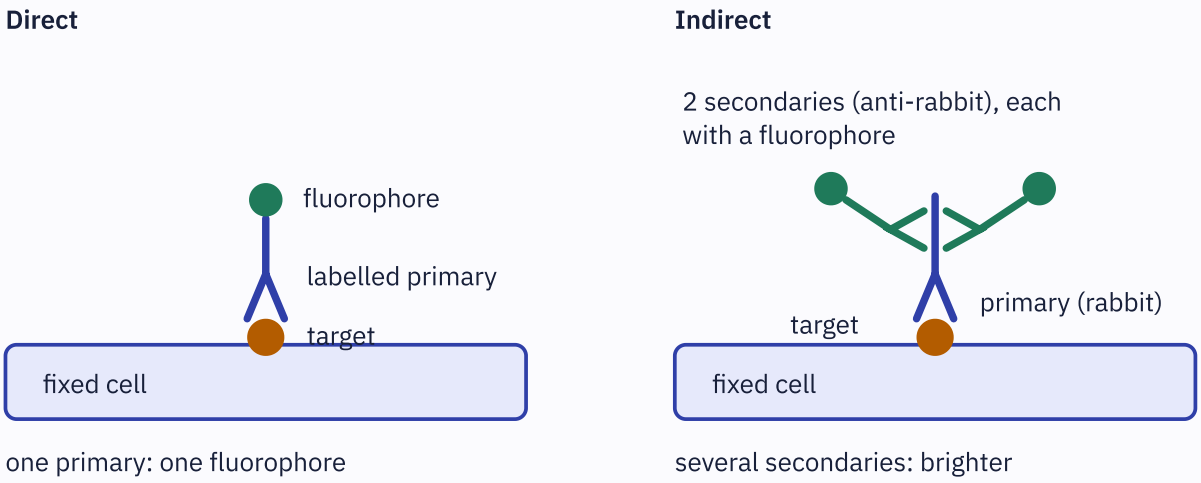


Figure 6.8.1 Direct against indirect immunostaining (antibodies drawn as Y shapes, not to scale). Each antibody grips its target with the tips of its arms. In the indirect method labelled secondary antibodies grip the stem (Fc) of the primary, which amplifies the signal.

Immunohistochemistry and histochemical staining

In **immunohistochemistry (IHC)**, used in pathology on tissue sections, the secondary antibody carries an enzyme, usually horseradish peroxidase (HRP). HRP with its substrate DAB (diaminobenzidine) and hydrogen peroxide gives a brown precipitate where the target is; the section is counterstained with haematoxylin (nuclei blue) and viewed in bright-field. Breast cancer biopsies are routinely stained this way for oestrogen receptor and HER2. Plain **histochemical stains** work without antibodies: haematoxylin and eosin (H&E) shows nuclei purple-blue and cytoplasm pink; periodic acid–Schiff (PAS) stains glycogen and mucins magenta.

Finding where a protein lives

Three methods support one another:

- **Co-localisation** with organelle markers: stain your protein green and a known marker red, for example TOM20 (mitochondria), calnexin (ER), GM130 (Golgi) or LAMP1 (lysosomes). Overlap shows yellow in a merged image, and a correlation value such as Pearson's coefficient (from −1 to +1) puts a number on it. Use confocal images, since widefield blur creates false overlap.

- **Fluorescent protein fusions**: fuse GFP to your protein's gene and watch it in living cells, including how it moves. Check that the tag does not hide a targeting signal, such as an N-terminal mitochondrial presequence.
- **Fractionation and Western blot**: separate nuclear, mitochondrial, membrane and cytosolic fractions (Chapter 6.6), run each on a gel and probe with your antibody plus marker antibodies.

WORKED EXAMPLE 1: DESIGN AN EXPERIMENT

Question (8 marks). A new human protein X is suspected to sit in mitochondria. You have a rabbit anti-X antibody. Design an experiment to test this and say what result would support it.

1. Grow cells on coverslips; fix in 4% paraformaldehyde for 15 min; permeabilise with 0.1% Triton X-100; block with 5% goat serum.
2. Primary antibodies together: rabbit anti-X and mouse anti-TOM20 (mitochondrial outer membrane marker).
3. Secondaries: goat anti-rabbit with a green dye; goat anti-mouse with a red dye. Counterstain DAPI.
4. Controls: secondaries only; cells with X knocked down by siRNA.
5. Image by confocal microscopy in separate channels to avoid bleed-through.
6. Support: green and red overlap in thread-like networks (yellow in the merge), Pearson's coefficient near +1, and the signal falls after knockdown. Confirm by fractionation: X should appear in the mitochondrial fraction with TOM20 in a Western blot, not with the cytosolic marker LDH.

WORKED EXAMPLE 2: DILUTING AN ANTIBODY

Question (2 marks). You need 5 mL of primary antibody at 1:500. How much stock and how much blocking buffer?

1. Stock volume = 5 mL ÷ 500 = 0.010 mL = **10 µL**.
2. Buffer = 5,000 µL − 10 µL = **4,990 µL**.

EXAM TRAPS

- The secondary antibody binds the primary antibody's Fc region, not the target protein.
- Aldehyde-fixed cells need a separate permeabilisation step; methanol fixation already permeabilises.
- The secondary must be raised against the primary's host species: a rabbit primary needs an anti-rabbit secondary.
- Overlap in a widefield image is weak evidence; confocal sections plus a number (Pearson's) and a second method are expected.
- Immunofluorescence uses fluorophores; IHC usually uses HRP–DAB and a bright-field microscope.

CHECK YOURSELF

– REMEMBER **Name the substrate that HRP turns into a brown precipitate in IHC.** 1 mark

DAB (3,3′-diaminobenzidine), with hydrogen peroxide.

– REMEMBER **Give one marker protein each for mitochondria, ER and Golgi.** 3 marks

TOM20 (mitochondria), calnexin (ER), GM130 (Golgi).

– UNDERSTAND **Why does indirect staining give a brighter signal than direct staining?** 2 marks

Several labelled secondary antibodies bind each primary antibody, so each target carries more fluorophores.

– UNDERSTAND **What is the purpose of the blocking step?** 2 marks

Proteins such as BSA or serum coat non-specific binding sites, so antibodies stick only to their epitope and background falls.

– APPLY **A secondary antibody is used at 1:1000 in 20 mL. How much stock?** 1 mark

20 mL ÷ 1000 = **20 µL** in 19.98 mL buffer.

– APPLY **You see bright staining in the secondary-only control. What does it mean and what will you change?** 3 marks

The secondary binds non-specifically, so the real staining cannot be trusted. Block longer or with serum of the secondary's host, dilute the secondary more, wash more, or change the secondary.

– ANALYZE **A GFP fusion of protein Y shows cytosol, but antibody staining shows mitochondria. Give two explanations.** 4 marks

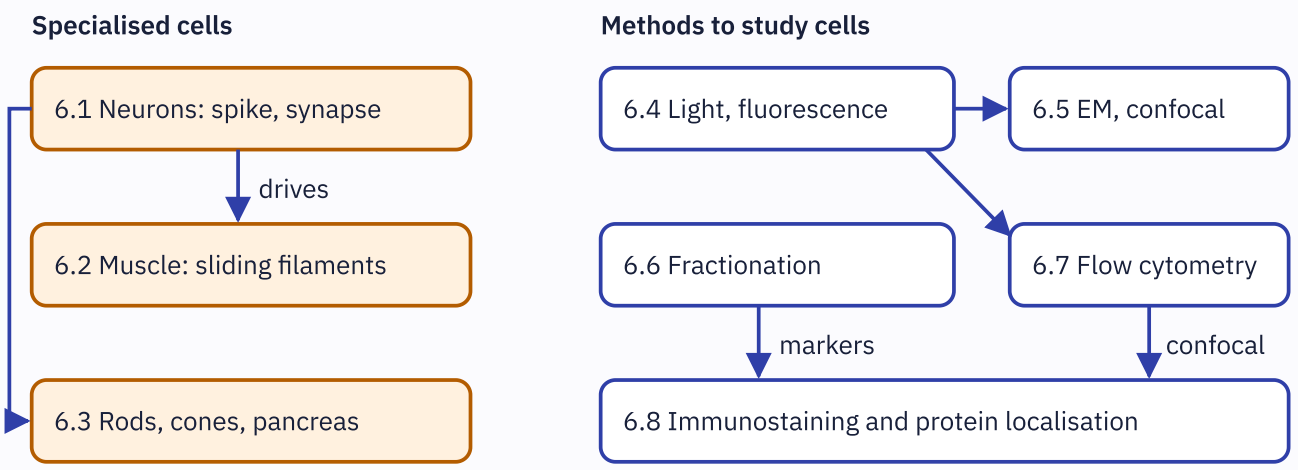
(1) GFP on the N-terminus masked the mitochondrial targeting presequence, so the fusion stays in the cytosol; try a C-terminal tag. (2) The antibody cross-reacts with a mitochondrial protein; test it in knockout cells and by Western blot of fractions.

BEYOND THE SYLLABUS

Not for the exam. The Human Protein Atlas has mapped the subcellular location of most human proteins by immunofluorescence and makes the images free online. Proximity labelling (BioID, APEX) tags neighbouring proteins with biotin in living cells to map compartments by mass spectrometry. Multiplexed imaging stains 40 or more proteins on one tissue section, now used in cancer research. Search next: "Human Protein Atlas subcellular" and "proximity labelling APEX".

Unit summary

TERM	MEANING OR FORMULA	CHAPTER
Nernst equation	$E = 61.5\ mV \times \log_{10}(C_{out} / C_{in})$ for a +1 ion at 37 °C	6.1
Action potential	All-or-none spike: Na^+ in (to about +30 mV), then K^+ out; threshold about −55 mV	6.1
Saltatory conduction	Spike jumps between nodes of Ranvier in myelinated axons	6.1
Sarcomere bands	A band = thick filament (constant); I band = sarcomere − A band; H zone = sarcomere − 2 × thin filament	6.2
Cross-bridge cycle	ATP binding detaches myosin; Pi release drives the power stroke	6.2
Phototransduction	Light → rhodopsin → transducin → PDE6 → cGMP falls → channels close → hyperpolarisation	6.3
β-cell coupling	Glucose → ATP/ADP up → K_ATP closes → depolarisation → Ca^{2+} in → insulin exocytosis	6.3
Resolution (Rayleigh)	$d = 0.61\ \lambda / NA$; $NA = n \sin \theta$	6.4
Haemocytometer	cells/mL = mean count per large square × 10^4 × dilution factor	6.4
Electron wavelength	$\lambda \approx 1.226 / \sqrt{V}$ nm (non-relativistic)	6.5
RCF	$RCF = 1.118 \times 10^{-5} \times r(cm) \times rpm^2$	6.6
Enrichment and yield	Enrichment = SA fraction ÷ SA homogenate; yield = units fraction ÷ units homogenate × 100%	6.6
FSC and SSC	Forward scatter ≈ size; side scatter ≈ granularity	6.7
Indirect immunostaining	Unlabelled primary + labelled secondary against the primary's Fc	6.8



The methods on the right are how the cells on the left were worked out.

Figure 6.S.1 How the eight chapters connect. On the left, nerve drives muscle, and photoreceptors are neurons too. On the right, the light microscope leads to EM and confocal (resolution) and lends its fluorophores to flow cytometry; fractionation markers and confocal imaging both feed protein localisation.

1. A specialised cell expresses its own set of proteins and builds its shape around one job; the genome is the same in every cell.
2. Neurons signal by an all-or-none action potential (Na^+ in, K^+ out) and pass it on at synapses by Ca^{2+}-triggered exocytosis; myelin makes conduction fast and cheap.
3. All three muscle types use actin–myosin sliding; skeletal and cardiac switch it on through troponin, smooth muscle through calmodulin and myosin light-chain kinase.
4. Light hyperpolarises photoreceptors through a G-protein cascade that lowers cGMP; β cells turn rising glucose into ATP, close K_ATP channels and release insulin.
5. Resolution depends on wavelength and numerical aperture ($d = 0.61\,\lambda / NA$), not on magnification; the light limit is about 0.2 µm.
6. Fluorescence relies on the Stokes shift and a filter cube; confocal pinholes remove out-of-focus light; electron microscopes reach nanometre detail but need dead, prepared samples.
7. Fractionation separates organelles by size (differential, rate-zonal) or density (isopycnic), checked by marker enzymes, enrichment and yield.
8. Flow cytometry measures scatter and fluorescence of single cells in thousands per second; immunostaining and co-localisation place a protein in its compartment.

Exam question bank

Remember and understand

– REMEMBER **Draw a labelled diagram of a myelinated neuron and state the function of each part.** 8 marks

Outline: dendrites (receive), soma with nucleus and Nissl bodies (synthesis), axon hillock (spike starts), axon (conducts), myelin by Schwann cells or oligodendrocytes (insulation), nodes of Ranvier (spike regenerated), terminals and synapse (transmitter release). Neat diagram with 7 or more labels.

– UNDERSTAND **Explain the ionic basis of the resting potential and of the action potential.** 12 marks

Outline: Na^+/K^+ pump (3 out, 2 in) sets gradients; K^+ leak gives rest near E_K; Nernst equation with values; threshold, Na^+ channel opening, peak, inactivation, K^+ efflux, undershoot; refractory period and one-way travel; all-or-none; saltatory conduction. A labelled voltage–time graph.

– UNDERSTAND **Compare skeletal, cardiac and smooth muscle cells, and describe the sliding filament mechanism.** 12 marks

Outline: comparison table (shape, nuclei, striations, control, junctions, Ca^{2+} switch); sarcomere bands and what changes; excitation–contraction coupling (T-tubule, DHP and ryanodine receptors, Ca^{2+}, troponin–tropomyosin); cross-bridge cycle with the role of ATP; relaxation by SERCA.

– UNDERSTAND **Describe the principle, parts and sample preparation of the transmission and scanning electron microscopes.** 12 marks

Outline: electron source, vacuum, electromagnetic lenses; transmission vs surface scanning; TEM preparation (glutaraldehyde, OsO_4, dehydration, resin, ultramicrotomy, uranyl acetate and lead citrate); SEM preparation (critical-point drying, sputter coating); resolution compared with light; images each is best for.

Apply

– APPLY **Derive the resolution limit of the light microscope and calculate it for λ = 500 nm with dry (NA 0.95) and oil (NA 1.40) objectives.** 8 marks

Outline: diffraction blur and Rayleigh criterion; d = 0.61 λ / NA, NA = n sin θ; dry: 0.61 × 500 / 0.95 = 321 nm; oil: 0.61 × 500 / 1.40 = 218 nm; why oil helps; empty magnification.

– APPLY **Outline a protocol to isolate mitochondria from rat liver and show how you would prove the fraction is pure. Include RCF calculations for a rotor of radius 7 cm.** 16 marks

Outline: cold isotonic sucrose buffer, Potter–Elvehjem homogeniser; 1,000 g pellet discarded; 10,000–20,000 g pellet kept; isopycnic gradient to remove peroxisomes and lysosomes; markers (SDH up, catalase, acid phosphatase, glucose-6-phosphatase down); specific activity, enrichment, yield; rpm for 1,000 g ≈ 3,600 and for 15,000 g ≈ 13,800 (rpm = √(RCF / (1.118 × 10^{-5} × 7))).

– APPLY **Describe the principle and instrumentation of flow cytometry and use it to measure apoptosis in drug-treated cells.** 12 marks

Outline: fluidics (hydrodynamic focusing), optics (laser, FSC, SSC, fluorescence filters), electronics (PMTs, pulses); gating and compensation; annexin V and PI principle; four-quadrant reading with a sample calculation; FACS sorting; controls.

– APPLY **Describe indirect immunofluorescence step by step to show that a protein is in the Golgi, with the controls you would include.** 10 marks

Outline: fixation and permeabilisation choices; blocking; primary anti-protein plus anti-GM130 from different species; matching labelled secondaries; DAPI; confocal imaging; merge and Pearson's coefficient; secondary-only and knockdown controls; confirm by fractionation and Western blot.

Analyze and evaluate

– ANALYZE **Compare widefield fluorescence, confocal and electron microscopy for studying the mitochondrial network of a living cell and its cristae. Which would you choose for each question?** 10 marks

Outline: widefield is fast and gentle but blurred in thick cells; confocal gives optical sections and 3D of the live network (with a mitochondrial dye or GFP), at the cost of bleaching; cristae (tens of nanometres) need TEM of fixed sections. Choose confocal for the network, TEM for cristae; mention resolution values.

– ANALYZE **Neurons, muscle cells and β cells all use Ca^{2+} to trigger their output. Compare where the Ca^{2+} comes from and what it triggers in each.** 12 marks

Outline (spans 6.1–6.3): nerve terminal, voltage-gated Ca^{2+} channels from outside, vesicle exocytosis; skeletal muscle, Ca^{2+} from the SR via ryanodine receptors, binds troponin; smooth muscle, calmodulin and MLCK; β cell, voltage-gated channels after K_ATP closure, insulin granule exocytosis. Common theme: depolarisation → Ca^{2+} rise → output; the end-point differs.

– ANALYZE **Two methods place a protein in different compartments: fractionation says ER, immunofluorescence says cytosol. Evaluate the possible causes and design experiments to resolve the conflict.** 12 marks

Outline (spans 6.6 and 6.8): fractionation artefacts (protein sticking to microsomes, cross-contamination; check markers and salt washes); staining artefacts (antibody cross-reaction, fixation extracting membranes, widefield blur); tests: knockout control, a second antibody, GFP fusion with both N- and C-terminal tags, confocal co-localisation with calnexin, protease-protection assay of microsomes.

– ANALYZE **A classmate counts 80 cells per large haemocytometer square from a 1:20 dilution and reports 1.6×10^5 cells/mL. Find the mistake and give the correct value.** 8 marks

Outline: correct value = $80 \times 10^4 \times 20 = 1.6 \times 10^7$ cells/mL. The classmate multiplied by 10^2 instead of 10^4, likely from forgetting the 0.1 mm depth and the mm^3-to-mL conversion. Also discuss counting rules (cells on two borders only), viability by trypan blue, and why 4 or more squares are averaged.

Glossary

Action potential	A brief, all-or-none reversal of membrane voltage that travels along an axon or muscle fibre.
Annexin V	A protein that binds phosphatidylserine exposed on the outer surface of apoptotic cells.
Axon hillock	Where the axon leaves the cell body; the action potential starts there.
cGMP	Cyclic GMP; holds photoreceptor cation channels open in the dark.
Compensation	Correction for one dye's light spilling into another dye's detector in flow cytometry.
Confocal microscope	A point-scanning fluorescence microscope whose detector pinhole rejects out-of-focus light.
Cross-bridge	A myosin head bound to actin during contraction.
Dichroic mirror	A mirror that reflects short wavelengths and transmits longer ones.
Differential centrifugation	Spinning at rising speeds to pellet particles in order of size.
Epitope	The small part of an antigen that an antibody binds.
FACS	Fluorescence-activated cell sorting: a flow cytometer that also separates cells.
Haemocytometer	A counting chamber of known depth and grid area used to count cells.
Immunohistochemistry	Antibody staining of tissue sections, usually with an enzyme such as HRP that makes a coloured product.
Isopycnic centrifugation	Separation by density: particles stop where the gradient density equals their own.
Islets of Langerhans	Endocrine clusters in the pancreas containing α, β, δ and PP cells.
K_ATP channel	A K^+ channel closed by ATP; it couples glucose metabolism to insulin release.
Marker enzyme	An enzyme found in one organelle only, used to trace it in fractions.
Microsomes	Small vesicles formed from ER and other membranes during homogenisation.
Myelin	Wrapped membrane layers around an axon that insulate it.
Numerical aperture (NA)	$n \sin \theta$; the light-gathering power of an objective, which sets its resolution.
Photobleaching	Loss of fluorescence as light destroys the fluorophore.
Resolution	The smallest distance at which two points can be seen as separate.

Rhodopsin	The rod visual pigment: opsin plus 11-cis-retinal.
Sarcomere	The contractile unit of striated muscle, from one Z line to the next.
Secondary antibody	A labelled antibody that binds the Fc region of a primary antibody.
Side scatter (SSC)	Light scattered at about 90°, reflecting a cell's internal complexity.
Specific activity	Enzyme units per mg of protein; a measure of purity.
Stokes shift	The difference between excitation and longer emission wavelength of a fluorophore.
Svedberg (S)	Unit of sedimentation coefficient, 10^{-13} s.

Sources and further reading

- Alberts B. et al., *Molecular Biology of the Cell*, 7th edition (2022), W. W. Norton: chapters on visualising cells, analysing cells and molecules, and the cytoskeleton (from memory; check).
- Lodish H. et al., *Molecular Cell Biology*, W. H. Freeman: chapters on culturing, visualising and fractionating cells, and on nerve cells (from memory; check).
- Karp G., *Cell and Molecular Biology: Concepts and Experiments*, Wiley: chapter on techniques in cell and molecular biology (from memory; check).
- Cooper G. M., *The Cell: A Molecular Approach*, Sinauer/Oxford University Press: chapter on tools of cell biology (from memory; check).
- Hall J. E., *Guyton and Hall Textbook of Medical Physiology*, Elsevier: chapters on membrane potentials, skeletal muscle contraction, the retina and insulin (from memory; check).
- Wikipedia, "Cell fractionation": en.wikipedia.org/wiki/Cell_fractionation (overview of homogenisation and isotonic, cold buffers).
- Abcam, "Cell fractionation: a complete overview": abcam.com knowledge center (differential and density-gradient centrifugation, purity checks by Western blot).

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